CRANFIELD UNIVERSITY

NIK ISKANDAR PUTRA BIN SAMSUDIN

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APPLIED MYCOLOGY GROUP
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PHD THESIS
Academic Year: 2012-2015

Supervisor: PROF NARESH MAGAN, DSC
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This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

ABSTRACT

Fusarium verticillioides contaminates maize with the fumonisin group of mycotoxins for which there are strict legislative limits in many countries including the EU. The objectives of this project were (a) to examine the microbial diversity of maize samples from different regions and isolate potential biocontrol agents which could antagonize *F. verticillioides* and reduce fumonisin B_1 (FB_1) production, (b) to screen the potential biocontrol candidates using antagonistic interaction assays and different ratios of inoculum on maize-based media and on maize kernels to try and control FB_1 production, (c) to examine whether the potential control achieved was due to nutritional partitioning and relative utilization patterns of antagonists and pathogen, and (d) to examine the effects of best biocontrol agents on *FUM1* gene expression and FB_1 production on maize cobs of three different ripening stages.

The total fungal and bacterial loads on maize from Malaysia, Mexico and France showed that the largest populations were on the former samples because of its high water content, regardless of the selective media used. The main fungal genera were *Fusarium*, *Aspergillus* and *Penicillium* based on frequency of isolation on different selective media. Other zygomycetes (*Rhizopus*, *Mucor*) and common soil fungi (*Phoma*, *Kabatiella*) were also isolated. A mycotoxigenic strain of *F. verticillioides* (FV1) was isolated from Malaysian maize kernels, and its identity confirmed molecularly. FV1 was used in all subsequent experiments. The effect of a_w × temperature on FV1 growth and FB_1 production was determined and found to be similar to reference strains.

A total of six microbial strains were screened. Interaction assays, growth and FB_1 inhibition showed that BCAs 1, 4, 5 and 6 were effective. Studies of different pathogen:antagonist inoculum ratios showed that both on maize-based media and on maize kernels BCA1 (*Clonostachys rosea* 016) completely inhibited FB_1 production at 25:75 inoculum ratio at 0.98 a_w. BCA5 (a Gram-negative motile bacterium) was also effective, and utilized in subsequent studies. In all treatments, FB_1 production was higher at 0.95 a_w when compared to 0.98.

The mechanism of action was examined by comparing C-source utilization patterns in the Niche Overlap Index (NOI) and Temporal Carbon Utilization Sequence (TCUS) studies. It was found that a_w × temperature had significant impacts on C-source utilization patterns by BCA1 and BCA5 when compared with FV1. BCA1 and FV1 occupied similar niches at 0.95 a_w+30°C and 0.98 a_w+25°C. Despite repeated attempts, BCA5 was unable to grow in PEG 600 modified C-source media, possibly due to its toxicity effects.

Subsequently, studies were carried out on maize cobs of different maturities. To this end, a q-PCR method was used to examine effect of the BCAs on relative *FUM1* gene expression, and FB_1 production was quantified by HPLC-FLD. Temporal studies on maize kernels showed that optimum *FUM1* expression and FB_1 production occurred after 10 days at both 0.95 and 0.98 a_w. A_w × temperature had significant impacts on growth, *FUM1* gene expression and FB_1 production by FV1 on maize cobs of different maturities. When using 50:50 pathogen:antagonist inoculum ratio, BCA1 significantly reduced FB_1 levels on maize cobs by >70% at 25°C, and almost 60% at 30°C regardless of maize ripening stage. For BCA5, FB_1 levels on maize cobs were significantly decreased in some treatments only.

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"…bismillahir-rahmanir-rahim…"

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"...in God we trust, with Knowledge we progress, by Love we live..."

"…the Time spent, the Knowledge acquired,
the Wisdom attained and the subsequent
Thoughts herein documented
are affectionately dedicated to
my loving parents and siblings…"

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LIST OF ABBREVIATIONS

AOAC	Association of Official Analytical Chemists
BCA	Biological Control Agent
BLAST	Basic Local Alignment Search Tool
CFU	Colony Forming Unit
DG-18	Dichloran Gylecerol-18
DNA	Deoxyribo-Nucleic Acid
EFSA	European Food Safety Authority
ELISA	Enzyme-linked Immunosorbent Assay
et al.	*et alia* (Latin), translated as "and others"
EC	European Commission
e.g.	*exempli gratia* (Latin), translated as "for example"
EU	European Union
FAO	Food and Agricultural Organisation
FIM	Fumonisin-inducing Medium
HPLC	High Performance Liquid Chromatography
IARC	International Agency for Research on Cancer
i.e.	*id est* (Latin), translated as "that is"
ITS	Internal Transcribed Spacer
IUPAC	International Union of Pure and Applied Chemistry
MEA	Malt Extract agar
MMA	Milled-Maize agar
MRC	Medical Research Council
NA	Nutrient agar
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose agar
PMTDI	Provisional Maximum Tolerable Daily Intake
PROMEC	Programme on Mycotoxins and Experimental Carcinogenesis
UV	Ultra Violet
WHO	World Health Organisation

LIST OF SYMBOLS

-H	hydrogen group
-OH	hydroxyl group
%	percent
°C	degree Celsius
°F	degree Fahrenheit
µ	micro, prefix of a measurement unit (10^{-6})
A	Absorbance
a_w	water activity
g	gram, derived into ng, µg, mg, kg
k	kilo, prefix of a measurement unit (10^{3})
K	Kelvin
L	Litre, derived into µL, mL
M	molarity
m	milli, prefix of a measurement unit (10^{-3})
mw	molecular weight
n	nano, prefix of a measurement unit (10^{-9})
n	number
pH	potential Hydrogen, scale for acidity and alkalinity
ppb	part per billion, (µg/kg)
ppm	part per million, (mg/kg)
ppt	part per trillion, (ng/kg)
psi	per square inch, unit for pressure
rpm	rotation per minute
S	Svedberg unit
sp.	species (singular)
spp.	species (plural)
V	volt
v/v	volume over volume ratio
w/v	weight over volume ratio

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CHAPTER 1

General introduction, literature review and research objectives

1.1 GENERAL INTRODUCTION

Maize (*Zea mays* L.) is a major staple food consumed globally (Shewry, 2007). The importance of maize as cereals in human dietary consumption comes from its ability to provide macronutrients (*e.g.*, protein, fat, carbohydrate), micronutrients (*e.g.* vitamins, minerals) and dietary fibre which are essential for human growth, maintenance and optimal health (Topping, 2007). In the field, maize and other crops are constantly exposed to extensive insect damage, a wide array of plant diseases and climatic changes (*e.g.*, high-low humidity, temperature). These, and the fact that no commercial hybrid of maize is pathogen-resistant at present (except for Bt maize), render maize highly susceptible to fungal infection which consequently results in loss in yield and quality. As the third most important cereal grains after wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L.) in terms of production and human consumption throughout the world (Abbasian, 2012), infection of maize crops by a range of fungal pathogens (*e.g.*, *Aspergillus flavus*, *Fusarium verticillioides*, *F. graminearum*, *F. proliferatum*, *Alternaria alternata*) requires effective control measures.

Among the most prevalent mycotoxigenic fungi infecting maize in the field and during storage are members of the genus *Fusarium* especially *F. verticillioides* and *F. proliferatum*. *Fusarium verticillioides* (Sacc.) Nirenberg 1976 [*Giberella fujikuroi* (Sawada) Wollenw. mating population A; synonym *Fusarium moniliforme* (obsolete)] is a member of the section Liseola, and a prolific and dynamic pathogen of maize

world-wide (Desjardins, 2006). In the field, infection of *F. verticillioides* in the maize crops is asymptomatic at first, in which it survives as an endophyte and proliferates alongside the maize seedlings both within the spermosphere and rhizosphere. As maize growth progresses, the infection turns systemic where it infects almost every part of the maize plant giving rise to diseases and symptoms such as leaf blight (*i.e.*, rapid browning due to loss of chlorophyll; chlorosis), leaf spot, sheath spot, stalk rot and ear rot. This infection is made worse by horizontal contamination (*e.g.*, leaves or cobs damaged by birds and insects, airborne spores contaminating tassels) which eventually leads to infection of the maize kernels. Among the major pest insects is the European corn borer (ECB; *Ostrinia nubilalis*) which plays an important role in promoting *F. verticillioides* infections and in the consequent fumonisin contamination in maize grain in temperate areas (Blandino *et al.*, 2015). The exact mechanism for the interchanging of these roles (asymptomatic endophyte ↔ symptomatic pathogen) remains largely unknown, but it is highly dependent on a multitude of factors such as maize and pathogen genotype (Desjardins *et al.*, 2007), environmental conditions, water availability, pathogenic load/inoculum, and the presence of other phyllosphere fungi (Bacon *et al*., 2008).

Besides causing severe infection to the entire maize crop (systemic), *F. verticillioides* also produces the fumonisin group of mycotoxins as part of its metabolism. So far, 15 different fumonisins have been discovered with the most toxically potent being fumonisin B_1 (FB_1; Stockmann-Juvalla and Savolainen, 2008). In rats and mice, fumonisins are nephrotoxic (toxic to kidney), hepatotoxic (toxic to liver) and cardiotoxic (toxic to heart). Fumonisins are also the causative agent for equine leuko-

encephalomalacia (ELEM) in horses, and porcine pulmonary oedema (PPO) in pigs (Marasas, 2001). Although there have been no conclusive evidence as to fumonisins toxicity in humans, several health implications have been proposed such as neural tube defect in embryos (Blom *et al*., 2006), oesophageal cancer (Wild and Gong, 2009) and acute mycotoxicosis (Stockmann-Juvalla and Savolainen, 2008). Since almost 100% incidence of *F. verticillioides* in maize or maize-based products indicated contamination with fumonisins (Soriano and Dragacci, 2004), the risk of these reaching the consumers through poor management of the food/feed supply chains (Magan and Aldred, 2007a) is probable and a threat to food/feed supply in many countries.

Although in 1993 the International Agency for Research on Cancer (IARC) classified fumonisins as a Group 2B toxin (*i.e.*, possibly carcinogenic to humans; due to lack of toxicological data on actual human subjects), the World Health Organization (WHO) nevertheless outlined the provisional maximum tolerable daily intake (PMTDI) of fumonisins B_1, B_2, or B_3 individually or in combination at 2 µg/kg body weight (Gareis *et al*., 2003). Furthermore in 2007, the European Union (EU) amended the legislation pertaining to the maximum levels of *Fusarium* toxins in maize and maize-based products. In brief, the stipulated levels are: 4 µg/g (≈4 ppm) FB_1+FB_2 in unprocessed maize; and 1 µg/g (≈1 ppm) FB_1+FB_2 in maize intended for direct human consumption.

At present, there is a collective effort being taken to try and control mycotoxin contamination of staple food crops such as maize by the development of different control strategies (*e.g.*, physical, chemical, biological). The application of biological control using indigenous microbial inoculants (BCAs) is being increasingly recognized

as a cheaper, viable and eco-friendly alternative that limits the excessive use of synthetic chemical pesticides for which strict EU legislations exist (Charan *et al*., 2011). However, few approaches have been effective in controlling *F. verticillioides*-maize interactions mainly due to the ability of *F. verticillioides* to exist both in asymptomatic and symptomatic states when infecting maize. Furthermore, because of the limited relative humidity range over which many BCAs can grow, it is important to produce formulations of the BCA inoculum which can be applied in a similar way to a chemical. Thus, often BCAs are only being used as part of an integrated management system (*i.e.*, Integrated Pest/Pathogen Management; IPM).

As *F. verticillioides* exists in two states (endophyte and pathogen) in maize which subsequently causes two types of infection (systemic and localized) respectively, BCAs have been applied separately to the seeds to try and control the systemic infection, and to the ripening cobs to try and control the localized fungal proliferation and to a certain degree, the fumonisins contamination itself. Previous studies have also demonstrated the effective manipulation of ecophysiological factors (*e.g.*, available water, temperature) to control fungal pathogens and mycotoxin contamination in maize (Battilani *et al*., 2011), and also in wheat and grapes (Magan *et al*., 2010). There are also emerging ideas and suggestions on the potential of inhibiting the key genes involved in the biosynthetic pathway of mycotoxigenic fungi to try and inhibit/minimize the mycotoxin biosynthesis at transcriptional level. In 2011, Schmidt-Hedyt *et al*. showed the relationship between ecophysiological factors, transcriptional genes, and deoxynivalenol (DON) production between *F. culmorum* and *F. graminearum in vitro.* However, there has been no attempt so far to incorporate the ecophysiological

conditions and the application of a viable and potent BCA to inhibit one of the key genes in the fumonisin biosynthethic pathway (*FUM1*) in *F. verticillioides* to try and inhibit fumonisin production.

1.2 LITERATURE REVIEW

1.2.1 Maize; nutritional importance, human consumption and life cycle

Cereals such as maize (*Zea mays* L.), rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.) constitute a major staple food for a large part of the world's human population apart from legumes (*e.g.*, peas, beans) and meat (*e.g.*, beef, mutton; Shewry, 2007). Not only do they provide macronutrients (*e.g.*, protein, fat, carbohydrate) and micronutrients (*e.g.*, vitamins, minerals) required by humans for growth, maintenance, and optimal health (Topping, 2007), cereals also provide large amount of non-starch polysaccharides, also known as dietary fibre which include among others the arabinoxylans, β-glucans, pectins and arabinogalactans. These dietary fibres which consist primarily of cellulose, hemicellulose, pectin and lignin (Holguín-Acuña *et al.*, 2008) resist human digestive enzymes and function as a water-holding-capacity agent which when present at sufficient level in foods can reduce intestinal transit time.

Being a type of annual plant which is able to grow in almost every part of the world, maize is one of the most important staple raw commodities world-wide, and also a major source of starch. Ranked as the third largest planted crop after wheat and rice (Abbasian, 2012), production was up to 844,405,181 metric tonnes by 2010 (FAOSTAT, 2012). The demand for maize and maize-based food products is highly

likely to increase in the near future because of the increasing population pressure against limited production from available land, as well as the underlying limited labour which is the result of migration of productive rural populations to urban centres to secure better employment (Ngwira *et al.*, 2014).

Maize was first cultivated in the United States of America (New Mexico and Arizona) about 2,100 BC (Roney, 2009). After thousands of years of genetic evolution and selective breeding, many varieties of maize exist (*e.g.*, flint corn, dent corn, sweet corn, waxy corn). Sugar-rich varieties of maize are called "sweet corn" and usually grown for human food, while "field corn" varieties are typically used for animal feed. Although maize is technically considered as grain, its kernels are also used in cooking as vegetable (fibre) or starch (carbohydrate) and sugars (corn syrup). Apart from being utilized as a human food (20%) and animal feed (65%), maize also has a wide range of industrial uses (*e.g.*, manufacturing of plastics, fabrics, adhesives, component of microbial culture media, corn steep liquor, bio-ethanol; Fairfood, 2012).

Maize undergoes several growth stages before it reaches physiological maturity. At present, there are various systems used to describe the growth and developmental stages of maize. According to Lauer (1997), the Iowa State University System 1984 is the most commonly used system. The basic principle of this system is based on morphological indicators (*e.g.*, plant height, total leaf collars, total leaves appeared). The developmental stages are mainly separated into two groups; vegetative (V) and reproductive (R; or loosely termed as ripening). Tables 1.1 and 1.2 describe briefly each of the maize developmental stages while Figures 1.1 and 1.2 illustrate the maize plant

and maize ear (cob) structural anatomies respectively (adapted from DuPont Pioneer, USA; www.pioneer.com).

Table 1.1. Vegetative stages (V) in maize plant growth (adapted from Lauer, 1997).

STAGE	DESCRIPTION
VE : emergence	Seed is placed in the ground and begins absorbing water until it reaches 30% moisture level. The mesocotyl elongation pushes the growing coleoptile to the soil surface towards sunlight, and the radicle root (primary) and some lateral roots (side) develop from the seed to collect more water and nutrients.
V(n) : n^{th} leaf collar *n = number of leaf	$1^{st} – 5^{th}$ Leaves Rapid growth. Second leaf unfurls, followed quickly by others. Initial radicle root stops growing and lateral roots grow rapidly. $6^{th} – 9^{th}$ Leaves Roots creep deeper into the soil. Tassel and ear shoots begin to form. $10^{th} – 18^{th}$ Leaves Rapid growth. Ear shoots continue to grow and the tassel starts to show.
VT : tasseling	Vegetative growth has stopped with all branches of the tassel fully visible, and not held in by the upper leaves. Plants have extended all of their leaves and are near their maximum height. Energy is going towards ripening the kernels. Tassel is fully formed and emerged and begins to drop pollens and initiates fertilization. In a normal maize field, pollination can occur over a five to seven day window with it being concentrated most heavily for the first two or three days. An individual maize plant at peak pollination can release half million pollens per day.

Table 1.2. Reproductive stages (R) in maize plant growth (adapted from Lauer, 1997).

STAGE	DESCRIPTION
R_1: silking	Silks visible outside husks. Silks will capture falling pollens that fall from tassel. Silks emerge from the base to the tip in two to five days and remain receptive for up to two weeks. The silks will continue to elongate until fertilized. Captured pollen moves down to ovule (kernel), where pollination occurs. This process takes about 24 hours. Silks are pollinated within two to three days. Successful pollination depends on simultaneous silks emergence and pollination. Moisture stress at this time causes poor pollination and reduces yield.
R_2: blister	Occurs approximately 10-14 days after silking. Kernels are very small and white on the outside, and have a blistered appearance. Fluid filling the kernels is clear in colour. Silks begin to dry and darken to brownish colour. Kernels have ≈85% water content and will gradually decline until harvest.
R_3: milk	Occurs approximately 18-22 days after silking. Kernels begin to show yellowish colour on the outside. Cell division in the endosperm is complete and followed by cell expansion and starch-fill in the individual kernels. Fluid filling the kernels has a milky texture, which is caused by the accumulating starch. Kernels have ≈80% water content. Cobs gain more size and volume.
R_4: dough	Occurs approximately 24-28 days after silking. Ear begins to display a brighter yellow in colour. Starch in kernels continues to accumulate and begins to harden to a paste-like/dough consistency from its earlier milky texture. Cob begins to develop a reddish colour.
R_5: dent	Occurs approximately 35-42 days after silking. Kernels are dented. Water content of kernels begins to decrease to ≈55%. Starch in kernels continues to harden from dough stage to a much harder texture beginning at the top of kernels where a small hard white layer of starch is forming and down towards the cob. The line indicating the hard starch layer will advance toward the kernel base.
R_6: maturity	Occurs approximately 55-65 days after silking. Kernels achieve peak dry matter accumulation and weight (≈30-35% water content). Hard starch layer reaches the cob and a black/brown abscission layer (black layer) forms signifying end of kernels growth for the season.

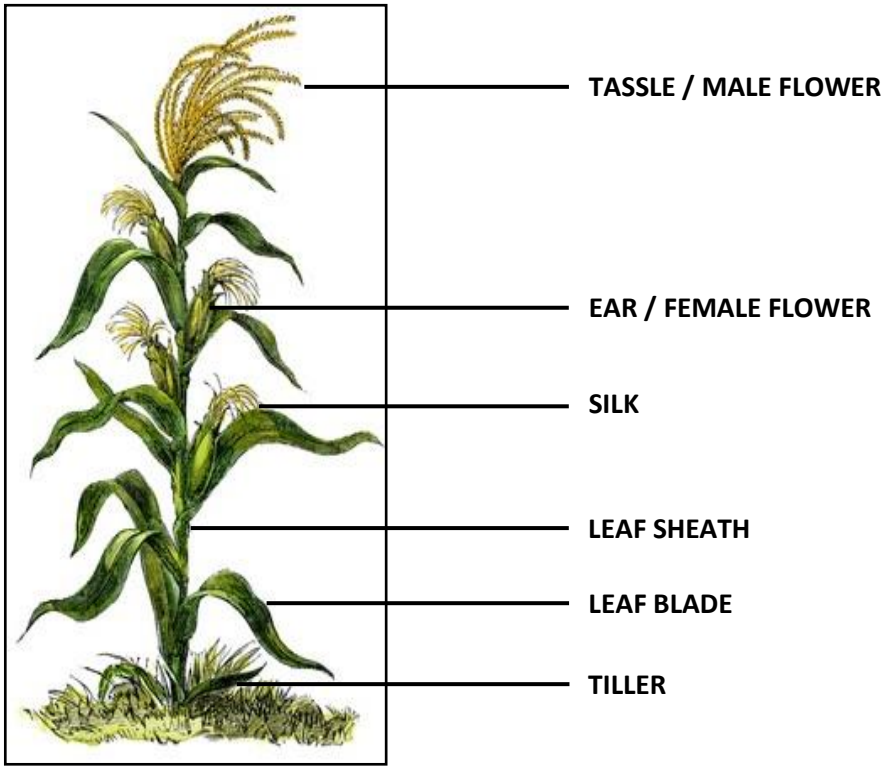

Figure 1.1. Maize plant structural anatomy.

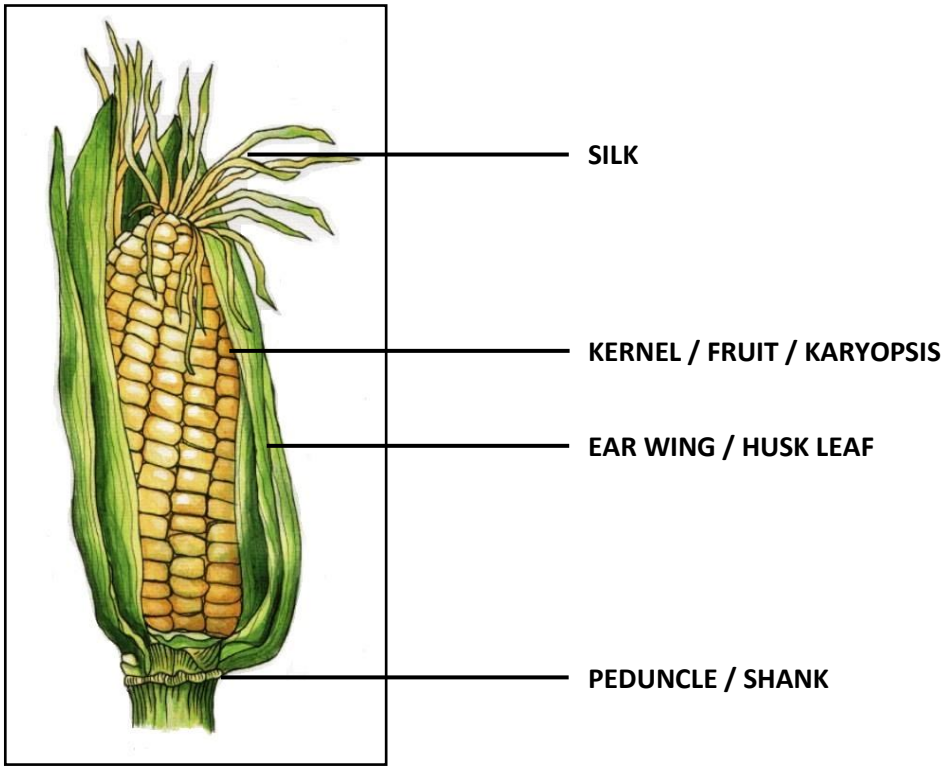

Figure 1.2. Maize ear (cob) structural anatomy.

The information and knowledge about the developmental stages of maize plant as well as its life cycle is essential in understanding the natural changes that regulate among other parameters, the nutrients (*e.g.*, carbohydrates, sugars, fatty acids) and water contents inside the kernels. This in turn provides a general idea on how the maize natural conditions from seedlings to physical maturity interact with the biotic (*e.g.*, insects, birds) and abiotic (*e.g.*, available water, pH, CO_2-O_2, temperature) factors which later collectively contributes to maize susceptibility to infection and diseases.

Over the years, natural susceptibility of maize to infection and diseases which result in global multi-million loss in yield and quality has prompted the scientific community to try and develop "better" maize. This "better" maize or genetically-modified (GM) maize has been genetically altered to express desirable traits physically (*e.g.*, bigger cobs, stronger roots and stems) and agriculturally (*e.g.*, resistance to pests, diseases, herbicides and drought) to generate high yields, increased value and reduced production costs. Several GM maize strains are presently being cultivated in various countries although its regulation varies between countries depending on intended uses (Wesseler and Kalaitzandonakes, 2011). In the USA, the 80 years of research and development in maize plant breeding is led by the Pioneer Hi-Bred International, Inc. (www.pioneer.com). However, developing a superior maize hybrid requires years of research (*e.g.*, conventional breeding, molecular breeding, in-bred selection), and multiple-generation field trials for hybrid performance and stability which would be approved by appropriate regulatory agencies before a specific hybrid could reach commercialization. Therefore, the abundance of information and knowledge on maize hybrids revolves around product development and hybrid performance (Tefera *et al.*,

2011), yields and resistance evaluation (Karavina *et al.*, 2014), and implications on disease management (Munkvold and Hellmich, 1999).

Among the highly prevalent cultivars of GM maize is the Bt maize which has been genetically engineered to express one or more proteins from the Gram-positive soil-dwelling bacterium *Bacillus thuringiensis*, hence the name. These Bt proteins are genetically expressed within the target plant (*e.g.*, maize, cotton) and will produce a highly potent toxin which is activated once it is ingested by the insect pests by paralyzing the insect's digestive system and forming holes in the gut wall and subsequently killing the insect through starvation (Hellmich and Hellmich, 2012). However, recent findings have alarmed farmers and consumers alike when cases of resistance in the pest insects were reported in Bt cotton in Gujarat, India (Bagla, 2010) and in Bt maize in Florida, USA (Marra *et al.*, 2012).

1.2.2 Infection and diseases in maize

In the field, plants are widely exposed to a range of environmental stresses (*e.g.*, abiotic, biotic) occurring at the same time, and different stresses can also occur at different stages of the plant's life cycle. Environmental stresses in plants are defined as a significant negative impact caused by non-living (abiotic stressors) and living (biotic stressors) environmental variables on the normal performance and physiological functions of plants (Vinebrooke *et al.*, 2004). Abiotic stresses range from water stress (drought, too little water; flooding, too much water); temperature stress (extreme high-low temperatures affect vital cell functions such as enzyme activity, cell division and membrane integrity); salt stress (high levels of salt in the soil can interrupt water and

nutrient uptake, reduce growth and photosynthetic activity); wind stress (strong wind can uproot plants by shear force and increase respiration rate through stomata) and pollutants (Tippmann *et al.*, 2006). Biotic stresses are inflicted upon plants by a wide array of living stressors (*e.g.*, bacteria, viruses, fungi, parasites, birds, insects, weeds; Redondo-Gómez, 2013). All these various environmental stressors which often exist simultaneously in an intricate web of cause and consequence can culminate in the infection and manifestation of diseases in plants.

Manifestation of diseases in maize in most cases is caused by biotic stresses while abiotic stresses (*e.g.*, warm and dry conditions) frequently provide a developing window of opportunity for infection to occur. For example, in the formation of *Fusarium* stalk rot, the climate does not directly initiate the disease but rather, the disease is actually caused by several *Fusarium* strains which thrive in warmer regions.

As an important source of food (in the forms of maize kernels), the severity of any infection or disease in maize is assessed by the loss of kernel quality (*e.g.*, kernel rot, cob rot), disruption of maize cultivation on the whole (*e.g.*, seed rot, stalk rot), and to a certain extent the accumulation of toxic substances (*e.g.*, aflatoxins, fumonisins) in the grains which are hazardous to human/animal health (Small *et al.*, 2012). While birds and insects indeed pose significant problems to maize cultivation and general health mostly by inflicting wounds and damages on maize plant surface, which subsequently become points of entry and breeding grounds for disease-causing microbes (Ofor *et al.*, 2009), this Subsection however will emphasize the other types of biotic stressors which are pathogenic microorganisms.

An updated and complete register for pathogenic microorganisms infecting maize is provided by The American Phytopathological Society (www.apsnet.org) which lists almost 150 types of infections and diseases caused by various bacteria, fungi, viruses and parasites. From the list, 75 symptoms and diseases are caused by a wide range of pathogenic microfungi followed by viruses (≈40), and bacteria and nematodes (≈15 each). Not only are the diseases in maize caused by a wide range of pathogenic fungi (*i.e.*, one strain infecting multiple plant parts, multiple strains infecting one plant part), but they also infect most parts of the maize plant as summarized in Table 1.3.

Table 1.3. Summary of some diseases and fungal pathogens in maize according to infection sites (adapted from The American Phytopathological Society, 2015).

MAIZE PART	DISEASES AND FUNGAL PATHOGENS
Seed	Seed rot (*Fusarium avenaceum*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium verticillioides*)
Seedling	Seedling blight (*Penicillium* spp., *Phomopsis* spp., *Pythium* spp., *Rhizoctonia* spp.)
Root	*Helminthosporium* root rot (*Helminthosporium pedicellatum*), *Pythium* root rot (*Pythium* spp.), *Rhizoctonia* root rot (*Rhizoctonia solani*)
Stalk	Stalk rot (*Fusarium* spp., *Glomerella* spp., *Pythium* spp., *Rhizoctonia* spp.)
Leaf	Leaf blight (*Colletotrichum graminicola*), banded leaf (*Rhizoctonia solani*), leaf spot (*Curvularia* spp., *Alternaria alternata*), leaf streak (*Diplodia macrospora*)
Ear	*Corticium* ear rot (*Corticium sasakii*), *Penicillium* ear rot (*Penicillium* spp.)
Kernel	Black kernel rot (*Lasiodiplodia theobromae*), *Cephalosporium* kernel rot (*Cephalosporium acremonium*), red kernel disease (*Epicoccum nigrum*)

However, there are also several fungal strains that are not only capable of infecting multiple maize parts, but also are known mycotoxin producers such as *Aspergillus flavus* which produces aflatoxins and causes *Aspergillus* ear and kernel rot (Bayman and Cotty, 1993; Bilgrami and Sinha, 1992; Cole and Cox, 1981; Stoloff, 1977), and also the more dynamic *Fusarium verticillioides* which produces fumonisins and inflicts symptoms on almost every part of the maize plant such as *Fusarium* ear rot, kernel rot, root rot, stalk rot, seed rot and seedling blight (Desjardins, 2006; Marasas, 2001).

1.2.3 *Fusarium verticillioides* in maize; life cycle and dynamics of infection

The genus *Fusarium* was first identified and described in 1809 by Johann H. F. Link then the director of the Botanic Garden in Berlin. Link characterized the genus by the "*fusiform*" shape (spindle-like; tapering at both ends) of its macroconidia which remains a characteristic trait of members of the genus. This genus is comprised of filamentous fungi which are widely distributed in soil and on plants. *Fusarium* species are usually a key component of the contaminating mycobiota of many perishable and durable commodities, especially cereals (Desjardins, 2006). Some species thrive in tropical (annual temperature mean of 18°C) and subtropical areas (annual temperature mean of 10°C), while others inhabit soil and contaminate crops in cooler climates.

Almost a hundred years later, in 1904, John Sheldon isolated a *Fusarium* strain colonizing maize kernels that was associated with animal toxicoses in Nebraska, United States, and named it *F. moniliforme* J. Sheldon. He derived the name from the Latin word "*monilia*" which means necklace *(i.e.,* distinctive long chain of microconidia; Sheldon, 1904). However, this specimen possessed irrefutable similarity to *Oospora*

verticillioides Sacc. (Saccardo, 1886) isolated earlier from maize in Italy but was described without the *fusiform* macroconidia which was diagnostic for *Fusarium* species. In 1976, Helgard Nirenberg rejected *F. moniliforme* and transferred *O. verticillioides* to *F. verticillioides* (Sacc.) Nirenberg, while retaining Saccardo as the original author, and the epithet "*verticillioides*" which described the whorled nature (*i.e.*, verticillate or cyclic) of the conidiophores (Nirenberg, 1976).

Taxonomically, *F. verticillioides* belongs to the Kingdom Fungi, Subkingdom Dikarya, Phylum Ascomycota, Subphylum Pezizomycotina, Class Sordariomycetes, Order Hypocreales, Family Nectriaceae, and Genus *Fusarium*. Among the many synonyms of *F. verticillioides* are *Oospora verticillioides* Sacc. 1886, *Alysidium verticillioides* (Sacc.) Kuntze 1898, *Fusarium moniliforme* J. Sheld. 1904, *Lisea fujikuroi* Sawada 1919, and *Gibberella fujikuroi var. fujikuroi* (Sawada) Wollenw. 1931. However, since the principle of "one fungus, one name" has been adopted in the International Code of Nomenclature for Algae, Fungi, and Plants, following the International Botanical Congress held in Melbourne in July 2011, after January 1st, 2013, one fungus can only have one name (Hawksworth, 2011), hence rendering the previous and less frequently used synonyms obsolete. The complete list of mating populations for *G. fujikuroi* species complex is shown in Table 1.4.

As an ascomycetous fungus, *F. verticillioides* reproduces predominantly asexually. The asexual stage is called the *anamorph* (*i.e.*, imperfect stage), and the sexual stage is called the *teleomorph* (*i.e.*, perfect stage). The teleomorph of *F. verticillioides* is *Gibberella moniliformis*. Generally, the asexual life cycle of *F. verticillioides* starts with

the vegetative reproductive spores (*i.e.*, conidiospores) which commonly contain a single nucleus as a product of mitotic cell divisions. These conidiopsores are genetically identical to the mycelium from which they originate. They are typically formed at the ends of specialized hyphae (*i.e.*, conidiophores, phialides). Most species in the genus *Fusarium* produce macroconidia, and many species but not all will produce microconidia (Samson *et al*., 2010). *F. verticillioides* produces small, hyaline, mostly single-celled microconidia which are abundantly produced in long catenate (*i.e.*, links) chains arising from morphologically simple phialides, and are highly adapted for wind, rain, and vectored dispersal as in the case of maize infection (Glenn, 2006).

Table 1.4. The mating populations for *Gibberella fujikuroi* species complex.

MATING POPULATION	SPECIES	REFERENCE
A and F	*F. verticillioides*	Leslie *et al.*, 1992
B and E	*F. subglutinans*	Elmer, 1995
C	*F. fujikuroi*	Xu and Leslie, 1996
D	*F. proliferatum*	Leslie *et al.*, 1996
G	*F. nygamai*	Leslie *et al.*, 1992
H	*F. thapsinum*	Klittich *et al.*, 1997

A: maize
B: sugarcane, millet
C: rice
D: maize, sorghum
E: maize (less frequently than A)
F: sorghum
G: rice (less frequently than C)
H: sorghum (less frequently than F)

Table 1.5 summarizes several major species of the genus *Fusarium*, type of plants they colonize, and mycotoxins they produce (Desjardins, 2006). Among the listed species, *F. graminearum*, *F. proliferatum* and *F. verticillioides* are the most predominant pathogens in maize which produce mycotoxins of agricultural and economic importance. The production of other biologically-active metabolites by members of this genus has also been described such as moniliformin (Marasas *et al.*, 1984) which is a cardiotoxic feed contaminant that causes ventricular hypertrophy (*i.e.*, the thickening of the ventricular walls in the heart) in fowls especially ducklings; fusaric acid (Bacon *et al.*, 1996) that can inhibit cell proliferation and DNA synthesis; and beauvericin (Logrieco *et al.*, 1998) which is active against Gram-positive bacteria and mycobacteria, and also capable of inducing programmed cell death in mammals. This Subsection however, will only discuss *F. verticillioides*, which unlike its sister species (*F. graminearum*, *F. proliferatum*) that colonize a wider range of hosts and only infect certain parts in maize, is able to infect the entire maize plant (Kuldau and Yates, 2000).

In maize, *F. verticillioides* is an excellent example of a facultative endophyte, in which, within the endophytic association with the maize, it can exist biotrophically (*i.e.*, harmless reliance on host for survival), as well as saprotrophically (*i.e.*, obtaining nutrients from decaying maize debris or soil). During the endophytic stage, *F. verticillioides* is transmitted vertically, through seeds, which initiates the asymptomatic (*i.e.*, without apparent symptoms) and systemic (*i.e.*, the whole plant) infection of maize.

Table 1.5. Selected mycotoxigenic species from the genus *Fusarium*, type of plants they colonize, and biologically-active metabolites they produce (adapted from Desjardins, 2006).

FUSARIUM SPECIES	PLANTS COLONIZED	MYCOTOXINS PRODUCED
Fusarium acutatum	Pigeon pea	Beauvericin
Fusarium crookwellense	Potato, Maize, Wheat	Fusaric acid, Nivalenol*, Zearalenone*
Fusarium culmorum	Barley, Maize, Wheat	Zearalenone*, Nivalenol*, Deoxynivalenol*,
Fusarium denticulatum	Sweet potato	Beauvericin, Moniliformin
Fusarium equiseti	Maize, Wheat	Zearalenone*, [1]Trichothecene*
Fusarium globosum	Maize, Wheat	Fumonisin*, Beauvericin
Fusarium graminearum	Barley, Maize, Wheat, Oat, Rice, Coffee, Potato	Deoxynivalenol*, Nivalenol*, Zearalenone*
Fusarium napiforme	Sorghum, Millet	Fumonisin*, Moniliformin
Fusarium nygamai	Sorghum, Pigeon pea, Rice, Bean	Beauvericin, Fumonisin*, Moniliformin
Fusarium poae	Barley, Oat, Wheat, Maize	Beauvericin, [1]Trichothecene*
Fusarium proliferatum	Asparagus, Banana, Barley, Maize, Wheat	Beauvericin, Fumonisin*, Moniliformin
Fusarium sacchari	Sugarcane	Beauvericin, Fusaric acid, Moniliformin
Fusarium solani	Tuber (Bean, Pea, Potato)	Moniliformin, [1]Trichothecene*
Fusarium sporotrichioides	Oat, Barley, Wheat, Millet	Moniliformin, T-2 toxin*, Beauvericin
Fusarium subglutinans	Maize	Beauvericin, Fusaric acid, Moniliformin
Fusarium verticillioides	Banana, Maize	Fumonisin*, Fusaric acid, Moniliformin

[1] Trichothecene type A (T-2 toxin, HT-2 toxin) and type B (deoxynivalenol, nivalenol)
If only trichothecene is mentioned, both types are produced
**** EU legislations for mycotoxins in food and feed exist***

Systemic infection starts with the fungal conidia or mycelia being carried inside the seeds or on the seed surface. *F. verticillioides* later develops inside the growing maize plant, moving from the roots to the stalk and finally to the cob and kernels (Oren *et al*., 2003). According to Wu *et al*. (2011), some conidia will colonize the veins of the maize root and grow along the veins after germination, while others will penetrate the plant cells, attach and form hyphae to penetrate neighbouring cells. Often, the mycelia will

migrate from root to stem inter-cellularly, while sometimes intra-cellularly. Figure 1.3 illustrates the infection cycle of *F. verticillioides* in maize.

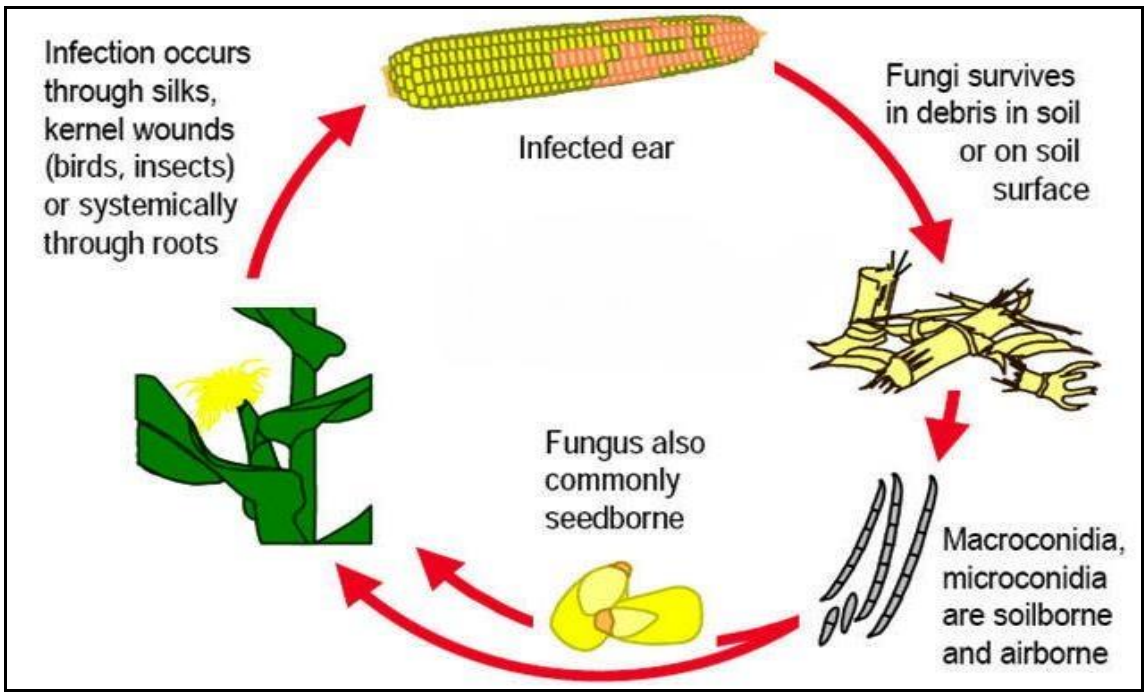

Figure 1.3. The infection cycle of *Fusarium verticillioides* in maize (adapted from DuPont Pioneer, USA; 2015).

Apart from being an endophyte which assumes asymptomatic association, *F. verticillioides* can also be a pathogen in maize which inflicts symptomatic and severe infection (Oren *et al*., 2003). The exact mechanism for this transformation remains elusive. However, environmental stresses (*e.g.*, low humidity, high temperature, nutritional availability; Velluti *et al*., 2000), host and pathogen genotypes, and presence of other phyllosphere mycobiota (Bacon *et al*., 2008) have been implicated.

As a pathogen in maize, *F. verticillioides* causes seedling blight, root rot, stalk rot, cob rot and ear rot (Gauperin *et al*., 2003). Seedling blight causes the seed or seedling to rot

and die. In the field, seedling blight is characterized by patches that do not germinate. Even if the seedlings do manage to germinate, they may not emerge, or they may emerge but die eventually. Stunted and yellow growth, or reduced root growth are the other symptoms for seedling blight. From the infected seeds, *F. verticillioides* moves upward to the maize stalks, causing the *Fusarium* stalk rot (pink stalk rot). This stalk rot is manifested by the rotting of the roots, lower stems and internodes in maize. It is slightly different from *Gibberella* stalk rot (red stalk rot) which is caused by *F. graminearum*. The stalk rot begins after pollination and develops into more severe rotting as the maize plant matures where the lower stem gradually turns soft, weak and shredded before collapsing (Khokhar *et al.*, 2014; Shekhar *et al.*, 2010).

F. verticillioides is also able to over-winter in infected maize residues as viable spores. During the growing season, these viable spores become airborne or can be rain splashed and will in turn infect the silks during flowering. Therefore, unlike the stalk rot that is transmitted vertically, *Fusarium* ear rot in maize is usually transmitted horizontally (Alakonya *et al*., 2008). It is by far the most common and damaging fungal-borne disease in maize occurring world-wide, in which it decreases yields, and grain quality, and also subjects the infected kernel to fumonisin contamination (Robertson-Hoyt *et al*., 2007). It is also known as cob rot, or kernel rot. Infected kernels exhibit whitish to pinkish or salmon-coloured mycelial growth (cultural characteristic of *F. verticillioides*). The fungal growth either consumes the entire cob (severe infection) or only partially. Figure 1.4 simplifies the dual-nature of *F. verticillioides* existence in maize.

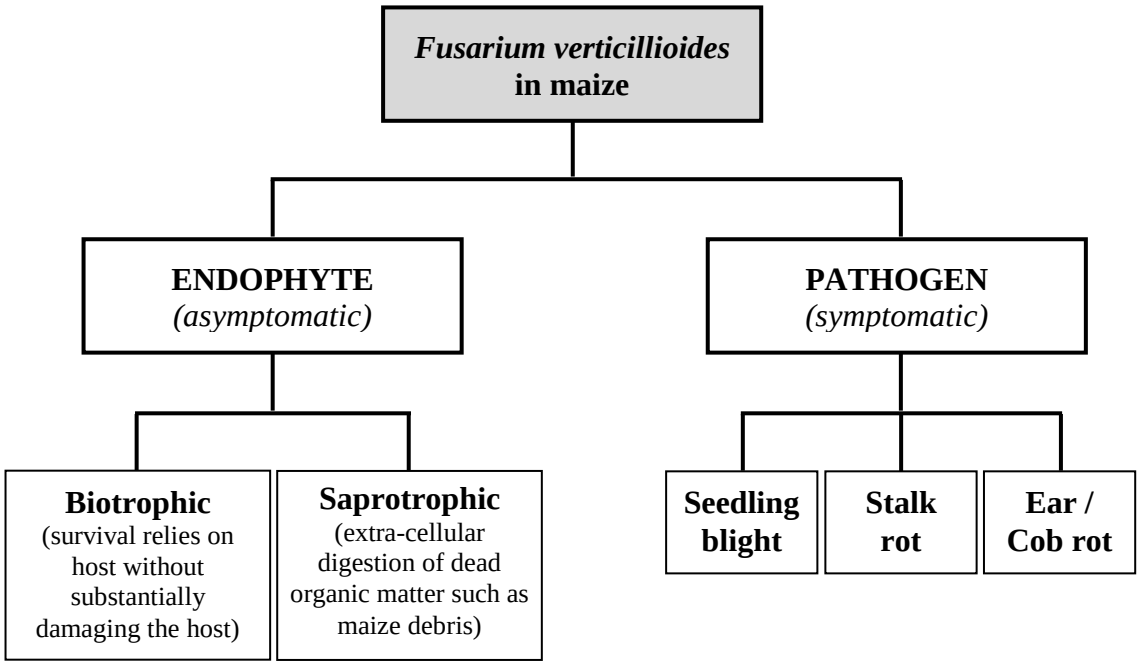

Figure 1.4. Summary of the endophytic and pathogenic nature of *Fusarium verticillioides* in maize.

1.2.4 Fumonisins; producton by *Fusarium verticillioides*, toxicity in humans and animals, and regulatory limits

Fumonisins are a group of foodborne carcinogenic mycotoxins primarily produced by several members of the genus *Fusarium* section Liseola (*e.g.*, *F. oxysporum*, *F. proliferatum*, *F. verticillioides*) in cereals such as maize and wheat. In 1988, the fumonisins were first isolated and chemically characterized (*i.e.*, type B_1 and B_2) as novel mycotoxins from cultures of *F. verticillioides* strain MRC 826 at the Programme on Mycotoxins and Experimental Carcinogenesis (PROMEC) of the Medical Research Council (MRC) in Tygerberg, South Africa, by Gelderblom *et al.* (1988) following a severe field outbreak of ELEM (equine leuko-encephalomalacia; a degenerative disease of the central nervous system that involves localized cerebral softening of the white matter of the brain) in horses. The outbreak was associated with contamination of horse

feed by *F. verticillioides* and can be fatal to horses after only a few days of consumption of contaminated feed. Animal feed prepared with maize kernels are commonly contaminated with FB_1 and FB_2 which have also been shown to cause porcine pulmonary oedema (PPO; fluid accumulation in the air spaces and parenchyma of the lungs that leads to impaired gas exchange and may cause respiratory failure) in pigs (Haschek *et al.*, 2001). In rat models, FB_1 has been shown to be highly hepatotoxic (*i.e.*, toxic to liver) and cardiotoxic (*i.e.*, toxic to heart). Hepato-carcinogenicity was also observed in rats where it causes primary hepatocellular carcinoma (*i.e.*, most common type of liver cancer) and cholangio-carcinoma (*i.e.*, a form of cancer originating in the bile ducts which drains bile from the liver into the small intestine; Marasas, 2001).

Fumonisins are named after *Fusarium moniliforme* (*i.e.*, the now obsolete synonym of *F. verticillioides*; Seifert *et al.*, 2003). Several series of fumonisins have been identified over the years which include the 1989 B-series; the C-series which lack the terminal methyl group of B-series and are mainly produced by *F. oxysporum*; the P-series which is a minor metabolite but closely related to B-series in which the amine group has been replaced by a 3-hydroxypiridinium and hence the name; and the A-series which are N-acetyl amides of B-series (Desjardins, 2006). A complete list of chemical structures for all the series is listed in Appendix A. Of the identified fumonisins, the B-series (Figure 1.5; B_1, B_2, B_3, B_4) are the most abundant in contaminated food and feed with the most toxically potent being the FB_1 ($C_{34}H_{59}NO_{15}$; Stockmann-Juvalla and Savolainen, 2008). Naturally, fumonisins are relatively simple long chain alcohols. Therefore, they have no UV-light-absorbing chromophore and hence, no fluorescence. Thus, fumonisins must be

derivatized for easy detection by high-performance liquid chromatography, usually by fluorescence detection of a suitable derivative of the free amine group (AOAC, 2002).

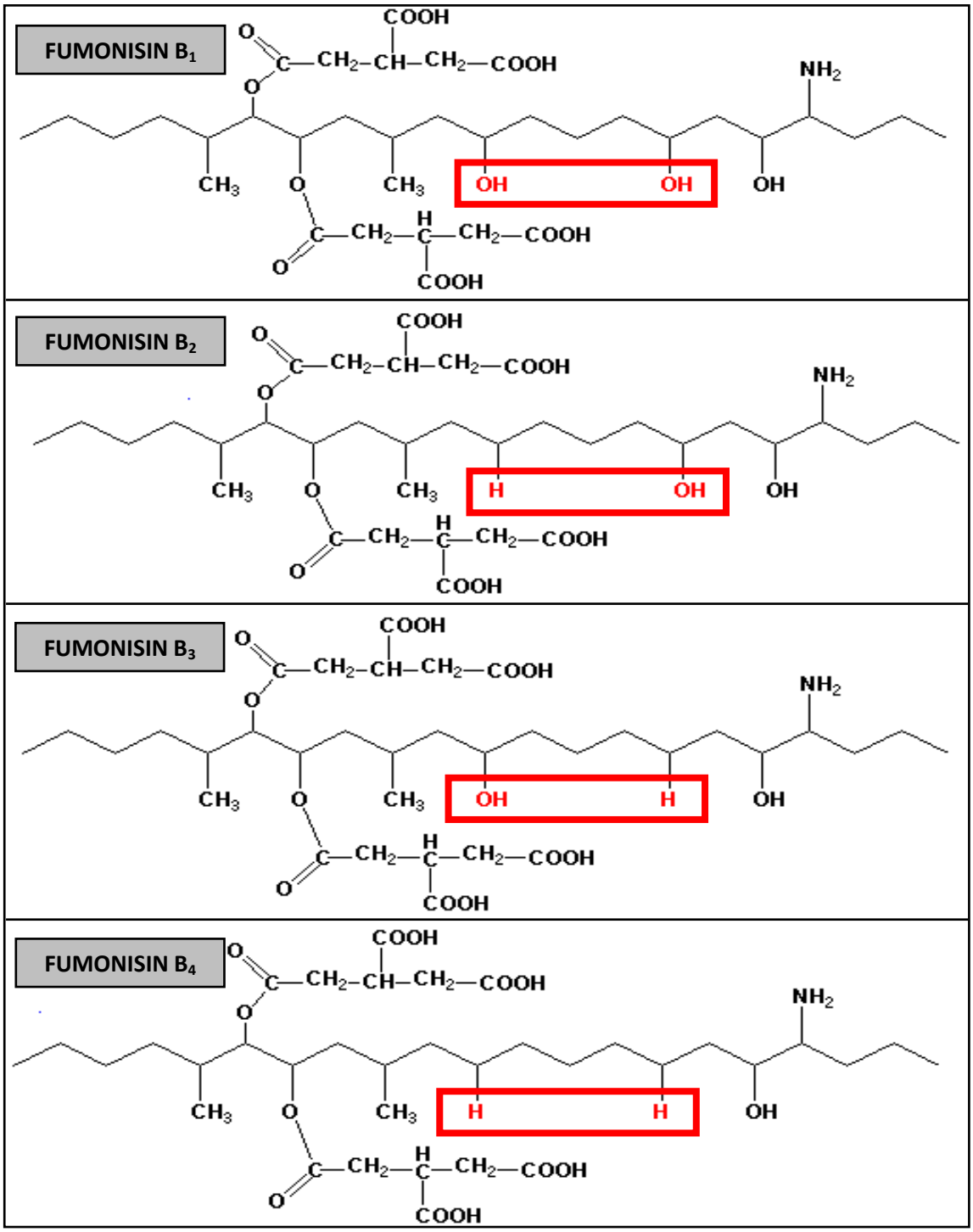

Figure 1.5. Chemical structures of B-series fumonisins (B_1, B_2, B_3, B_4) with different positions of the –H and –OH groups.

Even though elaborate toxicokinetics and toxicodynamics studies of the impacts of fumonisin on animal models have been performed, there has been no conclusive data implicating toxic effects of fumonisins on actual human subjects so far. Nevertheless, exposure of fumonisins to humans over the past decades which occurred at µg to mg levels at regions where maize products are the dietary staple (*e.g.*, African and American countries), have resulted in manifestation of at least three medical conditions in humans which based on epidemiological studies have been linked to *F. verticillioides* and fumonisins.

The earliest case study reported for human disease which was attributed to fumonisins as causal agent was oesophageal cancer in Kentani district of the Transkei region of South Africa in the 1950's where incident rates were significantly higher than that from other regions. Strong correlation was found between incidence of maize contamination by *F. verticillioides* and incidence of oesophageal cancer (Myburg *et al.*, 2002; Marasas, 2001). Significant correlation of high levels of fumonisins in maize to high rates of oesophageal cancer in rural populations of China has also been investigated and reported (Wild and Gong, 2009). Although the actual cause of oesophageal cancer remains unknown, *F. verticillioides* and fumonisins remain candidates of causal agents (Desjardins, 2006).

The second hypothesis of fumonisins toxic effect on humans is the manifestation of Neural Tube Defect in embryos (NTD; defect of the brain and spinal cord caused by failure of the neural tube to close) along the Texas-Mexico border around the same time FB_1 contamination of maize was implicated for ELEM in South Africa in 1989.

Because Mexican-Americans in the border region consumed maize-based tortilla, there was a high chance that they were exposed to high levels of fumonisins. As a matter of fact, NTDs were frequently recorded among births to Mexican-American women in 1990-1991 (Suarez *et al.*, 2000) and also in Cape Town area during 1975-1980 (Cornell *et al.*, 1983). Substantial evidences have pointed out that folate (*i.e.*, vitamin B_9 or folic acid) deficiency during pregnancy is a major risk factor for NTD in humans and animals (Blom *et al.*, 2006). In human intestinal cell lines, fumonisins generally disrupt sphingolipid metabolism and decrease its level in cell membranes. This disruption subsequently blocks folate uptake by the membrane-anchored major folate receptor, and hence, leads to the formation of NTD. High NTD incidence coinciding with high levels of FB_1 detected in maize also occurred in Guatemala and Transkei region of South Africa (Desjardins, 2006).

The third hypothesis is that fumonisins cause acute mycotoxicoses (*i.e.*, food poisoning by food products contaminated by fungi). In 1995, an outbreak of disease characterized by diarrhoea and abdominal pain occurred in 27 villages in India. The symptoms observed resembled that of an acute mycotoxicosis. It was later confirmed that excessive rain during that season brought about massive fungal contamination in maize which was used to make unleavened bread. Maize samples examined were highly contaminated with *Fusarium* spp. and contained high levels of FB_1 as compared to samples from unaffected households (Stockmann-Juvalla and Savolainen, 2008). Earlier in 1993, the International Agency for Research on Cancer (IARC) in Lyon, France, evaluated the toxins produced by *F. verticillioides* as Group 2B carcinogens (*i.e.*, possibly carcinogenic to humans; IARC, 1993). It is also noteworthy that further

research should be carried out to decisively implicate fumonisins as the cause of oesophageal cancer, neural tube defect, and acute mycotoxicoses in humans. Table 1.6 lists several important mycotoxins including fumonisins, and their respective levels of carcinogenicity towards humans according to groups (IARC, 2002).

Table 1.6. Classification of mycotoxins according to carcinogenicity towards humans (adapted from IARC, 2002).

GROUP	DESCRIPTION	MYCOTOXINS
Group 1	Carcinogenic to humans	Aflatoxins
Group 2A	Probably carcinogenic to humans	-
Group 2B	Possibly carcinogenic to humans	Fumonisins Ochratoxin A Sterigmatocystin
Group 3	Not classifiable as to its carcinogenicity to humans	Citrinin Patulin Zearalenone Tricothecenes*
Group 4	Probably not carcinogenic to humans	-

**** Trichothecene type A (T-2 toxin, HT-2 toxin)***
Trichothecene type B (deoxynivalenol, nivalenol)

In 2004, Soriano and Dragacci published a review on occurrence of fumonisins in foods world-wide. The food matrices examined were mainly maize and maize-based products. Fumonisins were found in the range of 0.01-140.4 mg/kg (1 mg/kg = 1 ppm). Assuming the average weight of ordinary maize consumer is 75 kg, there is an elevated risk that almost all consumers world-wide is consuming maize at a level exceeding the provisional maximum tolerable daily intake (PMTDI) of 2 µg fumonisins (alone or in combination) per kg body weight jointly prescribed by World Health Organization

(WHO) and European Commission (EC) (Gareis *et al*., 2003). Even though the toxic effects of fumonisins on humans (*e.g.*, oesophageal cancer, neural tube defect, acute mycotoxicosis) are yet to be conclusively determined, sufficient preliminary results are available to link those health conditions to fumonisins ingestion. Therefore, consuming fumonisins-contaminated maize or maize products above this level is clearly unadvisable and should be restricted.

It is also noteworthy that while the production of major (B-series) and minor (A, C, P-series) fumonisin analogues by *F. verticillioides* in maize and maize-based products have been extensively investigated, the occurrence of fumonisin-like compounds in maize and maize-based products have also been reported recently (Falavigna *et al*., 2012; Lazzaro *et al.*, 2012a). The contamination of these hidden fumonisins, which are usually found entrapped in maize macromolecules (*e.g.*, starch, protein), also poses a risk to food safety (Galaverna *et al.*, 2009).

Table 1.7 outlines the stipulated EU legislation [Commission Regulation (EC) No 1126/2007] for fumonisins in maize and maize-based food products (European Union, 2007). According to the Food and Agricultural Organization, regulations and legislation concerning mycotoxins in foods and feedstuffs have been introduced in over 120 countries world-wide (FAO, 2003). The European Union (EU) with its 28 member states probably is the strictest at enforcing the prescribed limits of mycotoxins. However, safety regulations on maximum permissible limits of fumonisins (mainly in maize) are only established by a handful of countries. FAO further reasoned that this might occur because of the lack in toxicological data, food consumption patterns,

distribution of fumonisins in food commodities, analytical methodology, and also commercial and trade interests.

Table 1.7. EU regulatory limits for fuminsins B (FBs) in maize and maize-based foods in µg/kg (adapted from European Union, 2007).

MAIZE PRODUCTS	SUM OF FB_1 AND FB_2 (µg/kg)*
Unprocessed maize, with the exception of unprocessed maize intended to be processed by wet milling	4,000
Maize and maize-based foods intended for direct human consumption	1,000
Maize-based breakfast cereals and maize-based snacks	800
Processed maize-based foods and baby foods for infants and young children	200
Milling fractions of maize with particle size > 500 mm, and other maize milling products with particle size > 500 mm not used for direct human consumption	1,400
Milling fractions of maize with particle size ≤ 500 mm, and other maize milling products with particle size ≤ 500 mm not used for direct human consumption	2,000

**** µg/kg is equivalent to part per billion (ppb)***
1,000 ppb is equivalent to one part per million (ppm)

1.2.5 Ecophysiology of *Fusarium verticillioides* during growth, maize colonization and fumonisins production

As *F. verticillioides* is among the major contaminants of maize, subsequent contamination of maize with fumonisins and other mycotoxins is potentially inevitable. Mycotoxins can contaminate the maize kernels in the field when environmental conditions favour fungal proliferation, and is made worse by the fact that these

mycotoxins can increase dramatically if storage conditions favour fungal growth and mycotoxin production (Desjardins, 2006). By understanding the ecophysiological factors involved during the maize-*F. verticillioides* life cycle, a better understanding of the pathogenic growth phases as well as the temporal and spatial nature of fumonisin production may be achieved.

Ecophysiology is the study of interrelationship between an organism's physical functioning (physiology) and its environment (ecosystem). Several environmental conditions (*i.e.*, abiotic components) such as temperature, water availability, pH, and gas balance between carbon dioxide (CO_2) and oxygen (O_2), govern the physiology of all organisms, including fungi. These abiotic conditions may themselves interact and impact on biotic components (*i.e.*, living organisms) to ensure the latter's growth and survival. Table 1.8 illustrates the ecophysiologically-based classification of fungi based on abiotic components.

The most important abiotic components that regulate the growth and fumonisin production by *F. verticillioides* are water activity (a_w), temperature (*T*), pH and gas composition and balance (Magan, 2007). The concept of a_w which was developed by Scott (1957) measures the amount of water readily available for microbial growth. The water content alone is not a totally reliable measure for water availability as some water is very tightly bound and relatively unavailable, and only the freely available water can actually be utilized by microorganisms. The concept further clarified that the a_w of pure water (H_2O) is 1.0 (equals to 100% equilibrium relative humidity; ERH) at a standard temperature and pressure. In most food commodities, there is a relationship between the

water content and a_w, and this is linked by the moisture adsorption curve. Each food matrix has its own moisture adsorption curve which entirely depends on the ability of the matrix to adsorb (adhesion to a surface) and desorb (release from a surface) water or more commonly known as the '*hysteresis effect*' which is further illustrated in Figure 1.6 (Chaplin, 2008).

Table 1.8. Classification of fungi based on ecophysiological components.

ECOPHYSIOLOGICAL COMPONENT	TYPE	DESCRIPTION
pH (Gross and Robbins, 2000; Nagai *et al*., 1995)	Acidophile	Grow at pH 2.0 – 5.0
	Acid-tolerant	Grow at pH 3.0 – 8.5
	Alkalophile	Grow at pH 7.0 – 10.0
	Alkali-tolerant	Grow at pH 5.0 – 10.0
Oxygen	Obligate aerobe	Only grow in the presence of oxygen
	Facultative aerobe	Can grow with or without oxygen
	Obligately fermentative	Growth by fermentation
	Obligate anaerobe	Cannot grow in the presence of oxygen
Temperature (Rajasekaran and Maheshwari, 1993)	Thermophile	Min 20°C, Max 50°C, Opt 40 – 50°C
	Thermotolerant	Grow in a wide range of temperatures
	Mesophile	10 – 40°C (most fungi)
	Psycrophile	Min 4°C, Max 20°C, Opt ≤16°C
Water availability (Clement *et al*., 1999)	Halophile	Grow at high salt concentration
	Osmophile	Grow at high osmotic pressure
	Osmotolerant	Grow at wide range of available water
	Xerophile	Can grow at available water < 0.80

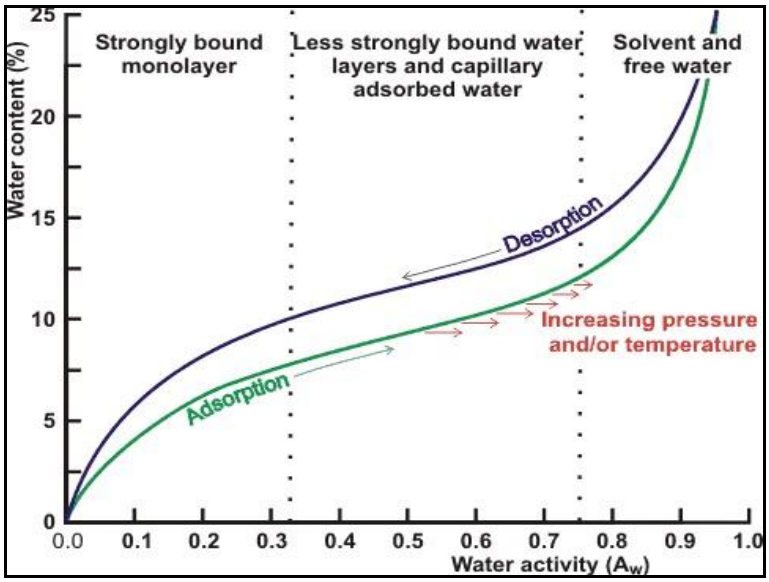

Figure 1.6. Example of a moisture adsorption curve and the hysteresis effect for maize (adapted from Chaplin, 2008).

The majority of fungi can grow between 0.900 – 0.995 a_w. However, xerotolerant and xerophilic fungi can grow with very little water down to 0.65 – 0.70 a_w. Many *Aspergillus* and *Penicillium* species are classified as xerotolerant or xerophilic (*i.e.*, able to grow at least in one part of its life cycle at ≤ 0.85 a_w; Pitt, 1975). In the case of *Fusarium* species, especially *F. verticillioides*, higher a_w levels are required (*i.e.*, 0.90 – 0.98 a_w) for growth which includes conidial germination, hyphal extension, and subsequently fumonisins production. The range of a_w for growth depends on the temperature at which *F. verticillioides* starts its life cycle (Marín *et al.*, 2004).

Another important abiotic component that has a major effect on the ecophysiology of *F. verticillioides* is temperature. Generally, temperature is defined as a measurement of the average kinetic energy of the molecules (internal thermal energy) in a matter (solid, liquid, gas) or system (light radiation), and can be measured with a thermometer or a

calorimeter. The International System of Units (SI) for temperature is Kelvin (K). Nevertheless, Celsius (°C) and Fahrenheit (°F) are also used interchangeably depending on the context of the subject matter, in which 0 Kelvin is equivalent to -273.15°C or -459.67°F. In microbial physiology, the role of temperature in fungal growth and life cycle is dictated to by its effects on metabolic activity, enzymes activity, and cellular permeability (Atlas, 1997). Temperatures for germination, growth, sporulation and mycotoxin production are different among fungi. Thus, minimum (inactivity of metabolism below this temperature), maximum (denaturation of major cellular proteins above this temperature), and optimum (highest growth rate at this temperature) temperatures are used to better elucidate the functional effects of temperature on fungal life cycle. Table 1.9 summarizes the specific conditions for a_w and temperature in the life cycle of *F. verticillioides*.

Table 1.9. Water activity and temperature effects on the life cycle of *Fusarium verticillioides*.

LIFE CYCLE	CONDITION (a_w, temp., media)	REFERENCE
Conidial germination	- 5°C (min), 25°C (opt), 37°C (max) - Min. a_w = 0.88 - 0.98 – 0.994 (all conidia germinate) - 0.92 a_w, 20°C (no germination) - 0.92 – 0.98 a_w, 30°C (hyphal growth)	Marín *et al.*, 1996
Hyphal growth	- Min. 2.5 – 5°C, 0.90 a_w - Opt. 22.5 – 27.5°C, 0.993 a_w - Max. 32 – 37°C	Wilson and Griffin, 1975 Joffe *et al.*, 1973
Sporulation	- Max. 30°C	Melcion *et al.*, 1998
Fumonisins production	- 10 – 37°C (irradiated corn) - 20 – 30°C (opt), 0.97 a_w	Marín *et al.*, 1999

Level of CO_2 is another ecophysiological factor that affects growth of microorganisms including *Fusarium* spp. As moulds are mainly facultative aerobes and highly sensitive to CO_2 (Smith *et al.*, 1990), the manipulation of CO_2 as a factor in controlling *F. verticillioides* colonization in maize and subsequent fumonisin production has been shown previously by Samapundo *et al.* (2007) who observed that the colony growth rates and maximum colony diameters of *F. verticillioides* and *F. proliferatum* decreased, whereas the lag phases increased, with the increase in CO_2 concentrations (*i.e.*, 10, 20, 30, 40, 60%). Differences in mycelial densities were also observed at higher CO_2 levels, with more sparse growth. This research provides new information in the modified atmosphere packaging (MAP) application for stored cereals. Any effect of CO_2 is linked to its solubility in water, which is higher than that of O_2, and leads to the formation of carbonic acid ($CO_2 + H_2O \leftrightarrow H_2CO_3$) which is toxic to most microorganisms and can lower the pH. Since CO_2 exists in the earth's atmosphere as a trace gas at 0.04 % (400 ppm) by volume in 2014 (Tans and Keeling, 2014), the effects of CO_2 on microbial growth and subsequent cellular functions is a part of climate-change-related research with implications that this may affect crop growth, including maize.

However, more information is required on the relationship between ecophysiological factors and the colonization of maize and fumonisin contamination by *F. verticillioides* during the different ripening stages of maize post-silking. There is also very little information on the ecophysiology under climate change conditions where combinations of drought stress, elevated temperature and two or three times existing CO_2 levels might occur as has been examined for *A. flavus* and aflatoxins (Medina *et al.*, 2014; Medina *et*

al., 2015). This is essential for the development of effective minimization or prevention strategies.

1.2.6 Expression of the *FUM* gene cluster and fumonisins production in *Fusarium verticillioides*

In general, secondary metabolism is a channel to store or discard unnecessary products generated during primary metabolism. The synthesis of these products, otherwise known as secondary metabolites, has been observed in plants, fungi, bacteria, algae, and animals (Hoffmeister and Keller, 2007). As these secondary metabolites are frequently produced at highest levels during the transition from active growth to the stationary phase, and the producer organism can grow in the absence of their production, it is suggested that secondary metabolism is not essential for short term survival. Furthermore, genes involved in secondary metabolism allow mutation and natural selection to obtain new beneficial traits. Secondary metabolism is also seen as an integral part of cellular metabolism and biology in which it relies on primary metabolism to supply the necessary enzymes, energy, substrates, and cellular machinery, and contributes to the long term survival of the organism (Roze *et al.*, 2011).

Since many secondary metabolites are produced at highest levels at specific times during the life cycle of an organism, it is believed that cells have developed a kind of "molecular signal" that activates the genes involved in secondary metabolism. This temporal signal will regulate at which time the pathway genes in secondary metabolism should be activated (Miller and Linz, 2006). Secondary metabolism also participates in

cellular defence (Schroeckh *et al.*, 2009), development (Keller *et al.*, 2005), and promotes survival during conditions of nutrient deprivation (Price-Whelan *et al.*, 2006).

In filamentous fungi, secondary metabolites are broadly divided into five diverse categories: polyketides, polyketide–peptide hybrids, fatty-acid-derived compounds, amino-acid-derived compounds and non-ribosomal peptides (Hoffmeister and Keller, 2007). The biosynthesis of polyketides requires the action of polyketide synthase enzymes (PKSs) which utilize a variety of acyl-CoAs (*i.e.*, group of coenzymes such as acetyl-CoA, propionyl-CoA, malonyl-CoA) as polyketides starter units. An example of such a fungal polyketide is fumonisin, which is primarily produced by members of the genus *Fusarium*, notably by *F. oxysporum*, *F. proliferatum* and *F. verticillioides* (Proctor *et al.*, 2013). Acetyl-CoA and the amino acids alanine, methionine, serine, and glutamic acid, are known precursors of fumonisins biosynthesis (ApSimon, 2001). With the partially established mechanism of spatial regulation, acetyl-CoA in filamentous fungi is formed either (1) in the cytosol, through conversion of cytosolic pyruvate into a cytosolic acetyl-CoA aided by pyruvate decarboxylase, cytosolic acetaldehyde dehydrogenase, and acetyl-CoA synthase (Boubekeur *et al.*, 2001), (2) in the mitochondria from β-oxidation of short-chained fatty acids or (3) in the peroxisomes from β-oxidation of long-chained fatty acids (Maggio-Hall *et al.*, 2005).

In 2001, four genes required for fumonisin biosynthesis in *F. verticillioides* were identified (Seo *et al.*, 2001). Subsequent mapping and characterization of the neighbouring genes resulted in the identification of 16 co-expressed genes located within the 42.5 kb region on chromosome 1 (Figure 1.7) which are responsible for the

complex biosynthetic pathway (Proctor *et al.*, 2003). *FUM1* encodes for a polyketide synthase (PKS) which is responsible for the formation of the polyketide backbone thereby catalyzing the initial step in the synthesis of the 20-carbon linear polyketide of fumonisins (Fanelli *et al.*, 2012). *FUM6* encodes for cytochrome P450 monooxygenase which catalyzes the electron transfer (reduction) of O_2 into –OH and water in the presence of NADPH ($RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$). *FUM7* encodes for alcohol dehydrogenase. *FUM8* encodes for pyridoxal phosphate-dependent aminotransferase which catalyzes the condensation of amino acids and acyl-coenzymeA. *FUM9* encodes for oxoglutarate-dependent dioxygenase which is responsible for the hydroxylation of the C-5 position (Butchko *et al.*, 2003). *FUM10* encodes for fatty acyl-coenzymeA synthetase. *FUM11* encodes for tricarboxylate transporters. *FUM12* encodes for monooxygenase which is responsible for the hydroxylation of the C-10 position. *FUM13* encodes for carbonyl dehydrogenase/reductase which reduces carbonyl to a hydroxyl group at C-3 position. *FUM14* encodes for peptide synthase condensation involved in tricarboxylic acid esterification at C-14 and C-15 positions. *FUM15* encodes for cytochrome P450 monooxygenase. *FUM16* encodes for fatty acyl-coenzymeA synthetase. *FUM17* and *FUM18* encode for longevity assurance factors which present *F. verticillioides* with self-protection and resistance to fumonisins. *FUM19* encodes for ATP-binding cassette (ABC) transporter which acts as efflux pumps to reduce cellular concentrations of fumonisins (Desjardins, 2006). *FUM21* encodes for cluster-specific transcription factor which belongs to the binuclear zinc cluster class of transcription factors, and is the primary candidate for a *FUM* gene cluster-specific transcription factor which controls the rate of transcription of genetic information from DNA to messenger RNA (mRNA)

by promoting (as an activator), or blocking (as a repressor) the recruitment of RNA polymerase (Brown *et al.*, 2007).

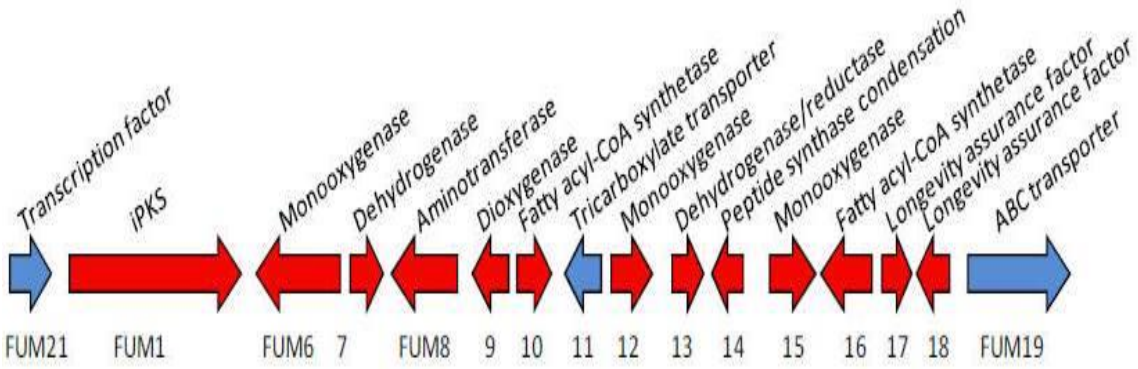

Figure 1.7. Fumonisin biosynthetic gene cluster (*FUM*) in *Fusarium verticillioides* genome (adapted from Proctor *et al.*, 2003). Arrows indicate gene size and direction of transcription. iPKS: iterative polyketide synthase; coA: coenzymeA; ABC: ATP-binding cassette, blue arrows: regulatory genes; red arrows: catalytic genes.

Although the *FUM* gene cluster has been completely sequenced and well characterized, few works have been carried out on its relationship with ecophysiological factors and the correlation with fumonisin production (Jurado *et al.*, 2008). Recently, some studies have examined the expression of *FUM* gene cluster and its significantly positive correlation with phenotypic fumonisins production under various ecophysiological conditions *in vitro* (Medina *et al.*, 2013; Lazzaro *et al.*, 2012b; Marín *et al.*, 2010; Jurado *et al.*, 2008). Nevertheless, more work is required to further elucidate the direct effect of these ecophysiological conditions combined with biocontrol applications *in vitro* and *in planta* on relative *FUM* gene expression, and how this is related to effects on fumonisins production by *F. verticillioides*. This is particularly important in relation to maize cobs during silking and subsequent storage.

1.2.7 Potentials for biological control of *Fusarium verticillioides* in maize

Different approaches within good agricultural practices (GAP) (*e.g.*, intercropping and plant rotation; application of pesticides/fungicides; selecting resistant gene pool during plant breeding programs) have been used to try and control infection by economically important plant pathogens and subsequent mycotoxin contamination in food crops (Strange and Scott, 2005). Physical, chemical, and biological controls have been utilized either individually or collectively before, during and after crop cultivation.

In physical control, mechanical methods such as seed selection, seed irradiation (*i.e.*, solar ray, gamma ray, ultra-violet ray) and ozonation (O_3) have been applied pre-cultivation of crops (Ruston, 1997) to remove or at least reduce pathogenic loads on seed surfaces. The use of barriers, netting and scarecrows to prevent attacks from rodents, insects and birds, controlled irrigation to minimize stresses on the crops, and early harvesting of crops contribute to physical control during crop cultivation (Vincent *et al.*, 2003). After crops cultivation or during post-harvest, sorting of visibly defective grains, warm-water washing, or de-hulling of grains especially in maize, were effective in achieving significant mycotoxin removal (Fandohan *et al.*, 2005).

In chemical control, the application of excessive synthetic chemical pesticides (*e.g.*, fungicide, herbicide, insecticide, molluscicide, nematicide) which contained phenolics, alcohols, halogens, heavy metals, or aldehydes (Marín *et al.*, 2008) and fertilizers are common in intensive farming practices aimed at high crop yield and quality (Zahir *et al.*, 2004). In controlling fungi, fungicides are primarily used. Fungicides are chemicals that kill yeasts and filamentous fungi, and are sometimes used as plant protection

product (PPP; European Union term for pesticides that are used to protect plants and plant products or to control unwanted plants). The fungicides are applied either on growing crops, or used as a post-harvest treatment to prevent fungi or moulds causing food to rot in storage or transport.

A Maximum Residue Level (MRL) which is the highest level of a pesticide residue that is legally tolerated in or on food or feed when pesticides are applied correctly (GAP) to protect animal and human health is also set for PPPs as part of the EU registration process. The MRL for pesticides in foodstuffs is 0.01 mg/kg (10 ppb). This general limit is applicable by default where an MRL has not been specifically set for a product or product type (European Union, 2005). Appendix B lists the fungicides as provided by the Health and Safety Executive, (HSE, 2015). Appropriate use of pesticides during crops cultivation could help in minimizing the phytopathogenic infection or insect infestation of crops and consequently mycotoxin contamination. However, these pesticides and fertilizers are generally expensive and will not be economically-viable for long-term application. Furthermore, the indiscriminate and excessive use of fungicides in crops is being discouraged due to increasing public awareness and growing concern on soil and water pollutions and the levels of residues present post-harvest. The recalcitrant, carcinogenic, and phytotoxic effects of many synthetic fungicides can also affect humans through the food chain. Moreover, excessive use of chemicals can result in the development of resistance in target fungal pathogens. The often long half-life of the fungicides in the environment, and the consumer awareness of residues on both perishable and durable commodities have resulted in a significant number of chemicals being removed from the approved list by the EU. This has thus been a driver for more

alternative control methods including IPM and biocontrol approaches which are less reliant on pesticide applications (Cabral *et al.*, 2013).

In biological control, living organisms are employed to inhibit, compete, or exclude the target pest or pathogen resulting in a reduction in numbers, or complete elimination (Janisiewicz and Korsten, 2002). In practice, biological control or simply biocontrol can be achieved by the introduction of a competitive microorganism which itself is harmless to the environment or niche in which it is introduced, but can inhibit or exclude the target pest or pathogen (Sharma *et al.*, 2009). Considerable success has been achieved in recent years in the application of biocontrol in combatting various infections and diseases in food crops in the laboratory and in the field (Lazarovits *et al.*, 2014; Mohale *et al.*, 2013; Nally *et al.*, 2012; Droby *et al.*, 2009).

Nevertheless, while biocontrol is an attractive approach that limits the use of excessive synthetic chemical fungicides, they have not been widely accepted commercially because of the lack of consistency and reproducibility in some formulations of the final product. Microorganisms employed in biocontrol must remain viable through the formulation process to effectively control pathogens under the range of fluctuating environmental conditions which can occur in nature. It has been shown that slight fluctuations in such abiotic factors can render a biocontrol agent ineffective for treatment of specific target fungal diseases (Singh *et al.*, 2011). There are also several bottlenecks in the application of biocontrol including: the limited relative humidity range over which many biocontrol agents can grow; production of viable formulations of the inoculum; and effective shelf-life similar to that of a chemical. Thus, often

biocontrol agents are being used as part of an Integrated Pest Management system (IPM; Clercq *et al.*, 2011).

Important criteria in evaluating the efficacy of biocontrol agents for minimizing mycotoxin production include (1) the ability to competitively colonize plant parts (phyllosphere) or root systems (rhizosphere) for nutrients or specific niches (Persello-Cartieaux *et al.*, 2003); (2) the ability to be viable under various ecophysiological conditions (*i.e.*, in the field or during storage) so that its growth and that of the pathogen coincide (Bacon *et al.*, 2001); (3) the ability to produce various antibiotic compounds, iron chelators and exoenzymes (*e.g.*, proteases, lipases, chitinases, and glucanases; Getha and Vikineswary, 2002); and (4) the compatibility with other control measures without inducing effects that compromise the end use quality of the commodity (Lugtenberg *et al.*, 2002). On the whole, the ability of biocontrol agents to control pathogenic fungi is dependent on the differential effects of macro- and micro-climatic conditions of the antagonist–pathogen interaction (Luongo *et al.*, 2005).

The use of biological agents to suppress growth of fumonisin-producing fungi in maize has been documented as early as 1998 when Desjardins and co-workers observed the inhibition of fumonisin formation by atoxigenic *F. verticillioides* strains (Desjardins *et al.*, 1998). However, higher incidence of ear and kernel rot was exhibited when the atoxigenic strains were inoculated during maize silking *in planta*. This observation implied that the ability to produce fumonisins is not required for a strain to cause ear or kernel rot. Even though the final goal was to exclude fumonisin-producing strains or at least prevent them from producing fumonisins, the manifestation of diseases at the end of

the experiment has paved the way for the search for novel non-disease-producing biocontrol agents.

A few years later, control of fumonisin-producing fungi in maize by rhizospheric and endophytic bacteria was reported (Bacon *et al.*, 2001). In general, these free-living rhizospheric and endophytic bacteria constitute a group of plant growth-promoting rhizobacteria (PGPR) which colonize the rhizosphere and produce substances to increase the growth of plants and/or protect them against diseases (Harish *et al.*, 2009). PGPR may protect plants against pathogens by direct antagonistic interactions with the pathogen, as well as through induction of host resistance. Members of the genera *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Herbaspirillum*, *Klebsiella*, *Pseudomonas*, *Rhizobium* and *Serratia* among others, have been found to enhance crop yields and protected certain cereals from fungal diseases including of maize and other crops (Chauhan *et al.*, 2015; Mehnaz *et al.*, 2010). Apart from the PGPR, some strains of yeasts and filamentous fungi have also showed promising antagonism under *in vitro* conditions against mycotoxigenic pathogens (Yates *et al.*, 2004).

Nevertheless, as effective and environmentally-friendly as biocontrol agents potentially may be, several commercial bioinoculants (*e.g.*, *Burkholderia* spp., *Pseudomonas* spp.) have been revoked and recalled from the market after questions were raised about the potential risk to humans both in terms of infection of users, or of immuno-compromised patients (Torbeck *et al.*, 2011). Thus, toxicology aspects are important, and certainly no growth at 37°C (the human body temperature) would be beneficial in any screening of potential microorganisms. These infections occur only in predisposed individuals who

have become heavily colonized by the bacteria which induce an inflammatory response in the host. Most often than not, these infections will lead to blood septicemia, and eventually death. Thus, screening programmes and efficacy assays must be done in this context as well as the down-stream processing steps to produce potentially useful commercial biocontrol formulations for commercial use (Chiarini *et al.*, 2006). Currently, several well-known antagonistic agents such as *Bacillus thuringiensis*, *B. subtilis*, *Pseudomonas fluorescens*, *Trichoderma* spp., and *Beauveria bassiana* have been exploited and commercialized as biopesticides for plant protection against various types of diseases. However, climatic and/or soil conditions or even different variety of target crops would influence the degree of control achieved in the field or indeed in store. Therefore, it has been suggested that it is important to isolate candidate antagonistic strains from the same environment as the pathogen as they may then be more likely to be effective as biocontrol agents (Cordero *et al.*, 2012; Card *et al.*, 2009).

In controlling *F. verticillioides*-maize interactions however, not many approaches are effective mainly due to the ability of *F. verticillioides* to exist both in symptomatic and asymptomatic states when infecting maize. The application of pesticides on maize plants will only eventually result in water and soil pollution and pesticide residues in crops without effectively eliminating the conidiospores of *F. verticillioides* within the spermosphere (seeds) and rhizosphere (roots). As *F. verticillioides* is an important pathogen responsible for both grain yield and quality losses in maize (Fandohan *et al.*, 2003), the biological control of colonization by this pathogen, and subsequent fumonisin contamination, has become an important focus for alternative control measures (Fareid, 2011). Since *F. verticillioides* exists in two states (endophyte and

pathogen) which subsequently cause two types of infection (systemic and localized), biocontrol agents need to either control seed-borne infection or during silking of maize cobs or by reducing the inoculum source on crop debris (Lyn *et al.*, 2009; Sivakumar *et al.*, 2000).

1.3 RESEARCH AIMS AND OBJECTIVES

The overall aim of the present work was to search for novel biocontrol agents to compete with and control the growth of *F. verticillioides* on maize and inhibit fumonisins production under different ecophysiological conditions. This required a good understanding of the ecophysiology of *F. verticillioides* and the nature of one of the key genes involved in the biosynthetic pathway of fumonisins (*FUM1*).

The present work was divided into four sequential Phases each with its own aims and objectives. The **first Phase** was to establish the fungal biodiversity on maize samples from different regions (Malaysia, Mexico, France) in terms of isolation frequency (% IF) and fungal populations (CFUs/g dry weight) for both *F. verticillioides* and other maize mycobiota. The **second Phase** was to identify some possible candidate microbial strains from Malaysian or other maize sources (bacteria, yeasts, filamentous fungi) which were antagonistic to the pathogen *F. verticillioides*. These studies were done in dual-culture assays with some of the indigenous strains and several known biocontrol strains which were obtained from other scientists. Antagonism was assessed from interaction scores in dual-culture assays and by measuring inhibition of *F. verticillioides* growth rate and colony development. This helped identify some potential effective candidates for further studies. The combined effects of varying inoculum ratios of

selected biocontrol agents (BCAs) on fumonisin B_1 (FB_1) production by *F. verticillioides in vitro* (3% milled-maize agar) and *in vivo* (gamma-irradiated maize kernels) under different a_w levels was then studied. Effects on FB_1 were quantified by HPLC-FLD. The **third Phase** involved studies on the possible mechanism of action of the best antagonists by using the Niche Overlap Index (NOI) approach and that of Temporal Carbon Utilization Sequence (TCUS) experiments. This was done to compare the nutrient consumption pattern of the pathogen and the antagonists relevant to maize which may elucidate their mechanisms of antagonism. The **fourth Phase** was designed to explore the efficacy of the best antagonists to control *F. verticillioides* on maize cobs of three different ripening stages by examining (a) *FUM1* gene expression as an indicator of fumonisin production and (b) actual FB_1 production on the maize cobs to evaluate the level of control which could be achieved at different ripening stages when using 50:50 pathogen:antagonist inoculum ratio after artificial inoculation of the cobs. The detailed objectives of all Phases are described below:

PHASE 1

Biodiversity of mycobiota in maize samples from different regions

(a) To establish the biodiversity of indigenous mycobiota in maize kernel samples from different regions (Malaysia, Mexico and France)

(b) To study the effects of water content and water activity of maize kernels on the mycobiota structure and populations

(c) To determine the isolation frequency (% IF) and total fungal load (CFUs/g dry weight) of the isolated strains

(d) To isolate and identify *Fusarium verticillioides* strains from the maize kernels

(e) To examine the effects of different ecophysiological factors (*i.e.*, different a_w levels and temperatures) on growth and fumonisin production by the isolated *F. verticillioides* strains on maize-based medium

PHASE 2

Efficacy of potential biocontrol agents for control of *Fusarium verticillioides* FV1 and fumonisin B_1 under different inoculum ratios and water activities on milled-maize agar and stored maize kernels

(a) To isolate indigenous bacteria and yeasts from Malaysian maize kernels as potential biocontrol candidates against *F. verticillioides* FV1

(b) To screen the isolated candidates as well as to test existing biocontrol candidates for antagonism against *F. verticillioides* FV1 *in vitro* in terms of interaction scores, daily growth rate (mm/day) and colony development (cm^2)

(c) To examine the best potential biological control agents (BCAs) by using different ratios of pathogen:antagonist (100:0; 25:75; 50:50; 25:75; 0:100) on milled-maize agar (*in vitro*) and on stored maize kernels (*in vivo*) under different a_w conditions on relative control of fumonisin B_1 (FB_1) production

PHASE 3

Carbon utilization patterns and niche overlap index between potential biocontrol agents and *Fusarium verticillioides* FV1 under different ecophysiological conditions

(a) To identify the similarity and differences in C-source utilization patterns by the potential biocontrol agents and *F. verticillioides* FV1 under different a_w × temperature conditions

(b) To use this data to obtain Niche Overlap Indices (NOIs) between the antagonists and the pathogen under different a_w × temperature conditions

(c) To determine whether there were differences in the rate of utilization of the C-sources in maize using the Bioscreen system to obtain the Temporal Carbon Utilization Sequence (TCUS) by the antagonists and the pathogen under different a_w × temperature conditions

PHASE 4

Effects of biocontrol agents in controlling fumonisin B_1 production by *Fusarium verticillioides* FV1 in maize cobs of different ripening stages by monitoring both *FUM1* gene expression and phenotypic toxin production

(a) To study the effects of incubation periods on *FUM1* gene expression and FB_1 production by *Fusarium verticillioides* FV1 on maize kernels

(b) To examine the effects of artificial point inoculation of antagonists and pathogen (50:50 ratio) on maize cobs of three different ripening stages and thus a_w levels, on *FUM1* gene expression using q-PCR and FB_1 production using HPLC-FLD

The overall overview of the project with regard to thesis arrangement is schematically represented in Figure 1.8.

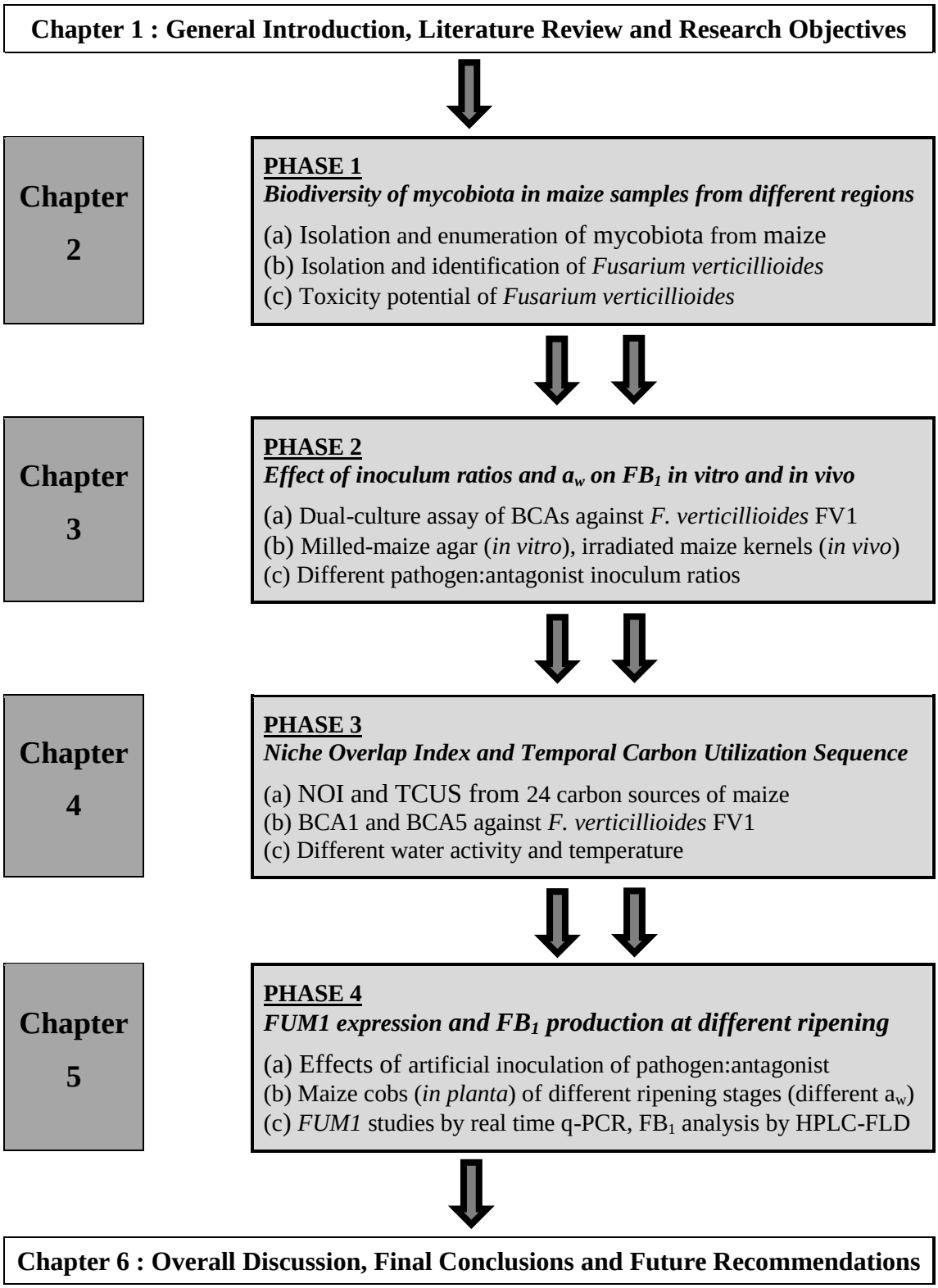

Figure 1.8. Flow diagram of experimental works and thesis arrangement.

CHAPTER 2

Biodiversity of mycobiota in maize samples from different regions

2.1 INTRODUCTION

By definition, "mycobiota" refers to the entire group of fungal flora present in a particular geographic region or habitat, deriving its modern coinage from the combination of two Greek words "*mukēs*" (*i.e.*, fungus) + "*biotē*" (*i.e.*, way of life). In plants, the mycobiota can be present either in the phyllosphere (*i.e.*, aerial plant surfaces) or the rhizosphere (*i.e.*, subterrestrial plant surfaces). These will interact with each other, with some being commensals, symbiotic or pathogenic. Like other food crops, the aerial plant surfaces of maize including ripening cobs are naturally inhabited by a wide range of fungal genera, the most prevalent being *Alternaria*, *Aspergillus*, *Penicillium*, and *Fusarium* (Lacey *et al.*, 1991; Venancio and Paterson, 2007; Reddy *et al.*, 2009). Other fungal genera are also present such as the Zygomycetes (*Mucor* spp., *Rhizopus* spp.), although they are less predominant (Atayde *et al.*, 2012). When the ecophysiological conditions are favourable, several members of these genera are capable of producing toxic metabolites such as aflatoxins by *Aspergillus flavus* (Giorni *et al*., 2007; 2011), fumonisins by *Fusarium verticillioides* and deoxynivalenol by *F. graminearum* (Desjardins, 2006).

Although the advancement in molecular biology and genetics may mean that identification of an organism is made faster and more accurate to species level, in actual practice, filamentous fungi are still mainly identified down to genus level by phenotypic characters, such as morphological and cultural characteristics primarily because this

method is less expensive and can avoid the multiple steps in molecular identification. However, it is also important to identify fungal strains to a species level as each species within a genus may have very different functional characteristics, such as mycotoxin profiles and ecophysiological properties (EFSA, 2007). In the present study, conventional morphology and cultivation techniques are complimented with molecular techniques (*e.g.*, 18S DNA, PCR, BLAST) to obtain both qualitative and quantitative information on the community structure and diversity of the mycobiota present in maize kernel samples from different regions.

Therefore, the objectives of this Chapter were:

(a) To establish the biodiversity of indigenous mycobiota in maize kernel samples from different regions (Malaysia, Mexico and France)

(b) To study the effects of water content and water activity of maize kernels on the mycobiota structure and populations

(c) To determine the isolation frequency (% IF) and total fungal load (CFUs/g dry weight) of the isolated strains

(d) To isolate and identify *Fusarium verticillioides* strains from the maize kernels

(e) To examine the effects of different ecophysiological factors (*i.e.*, different a_w levels and temperatures) on growth and fumonisin production by the isolated *F. verticillioides* strains on maize-based medium

2.2 MATERIALS AND METHODS

2.2.1 Sampling of maize kernels

Samples of maize kernels were obtained commercially from three different regions in 2012 (*i.e.*, Malaysia, France and Mexico). These were stored at 4°C, and isolations were made from replicate samples of these sources.

2.2.2 Measurement of water content and water activity of maize samples

The water contents of maize samples were measured by the oven-drying method. Three replicates of 10 g maize kernels of each sample were weighed on an analytical balance (Ohaus, Switzerland) with an accuracy of ± 0.01 g. The maize kernels were then transferred into a 100 mL beaker and dried in a drying oven (Genlab, UK) at 100 ± 1°C. The maize kernels were left to dry in the oven for 24 hours. At the end of this period, the samples were re-weighed, and the water content calculated using Equation 2.1.

$$\textit{\% water content} = \frac{\textit{weight before drying} - \textit{weight after drying}}{\textit{weight before drying}} \times 100$$

Equation 2.1. Formula to calculate water content.

However, the total water content alone is not a reliable indicator of that which is available for microbial colonization. This is because some of the water is very tightly bound to the components of the maize kernels and thus unavailable to microorganisms. The water activity (a_w) thus reflects that available water for microbial growth, and there is a relationship between the water content and a_w for different durable commodities.

Therefore, the a_w of the maize samples was also measured. To do this, a a_w meter (AquaLab, USA) was used (Figure 2.1). Since temperature affects the a_w directly, the samples were brought to ambient conditions (*i.e.*, 20-25°C) in the laboratory prior to measurement. The maize samples were placed in the plastic containers and introduced into the measuring chamber, and sealed. Once equilibrium was achieved (*i.e.*, within 5 min), the a_w and temperature reading was obtained. The a_w and temperature of three replicates of each maize sample were then measured.

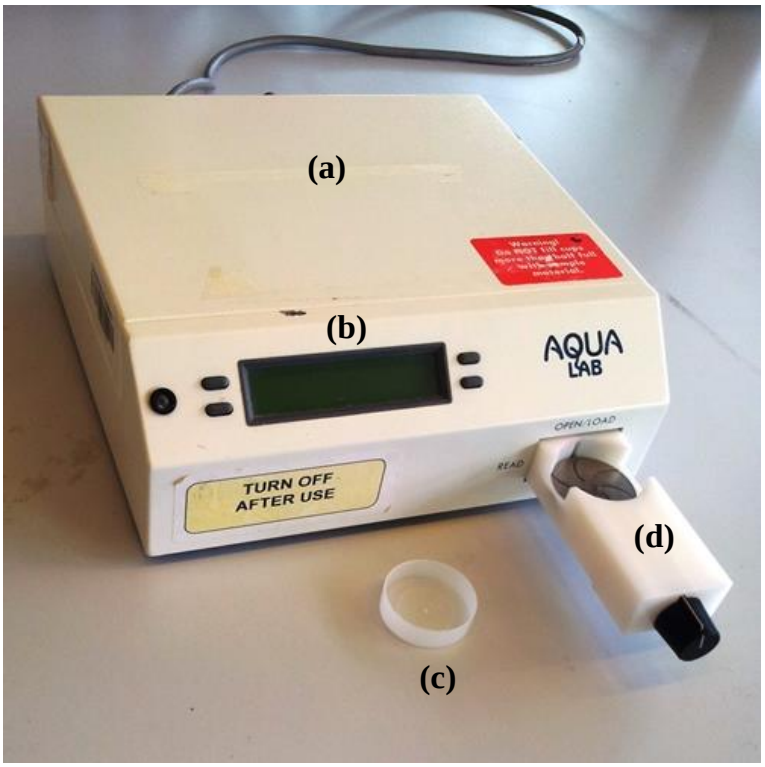

Figure 2.1. A water activity meter; (a) main unit, (b) digital display, (c) measuring cup, (d) measuring chamber

2.2.3 Preparation of growth media for microbial cultivation

Several types of growth media were used for fungal and bacterial cultivation. Malt Extract agar (MEA), Potato Dextrose agar (PDA), Nutrient agar (NA) and Dichloran-

Glycerol 18 agar (DG-18) were obtained commercially (Oxoid, UK). PDA, MEA and NA are non-selective media as they support the growth of a wider range of microorganisms. NA is commonly used for the routine cultivation, maintenance and enumeration of non-fastidious bacteria (*i.e.*, bacteria not having a complex nutritional requirement), and PDA and MEA for fungi. DG-18 however, is a selective medium which supports growth of xerotolerant/xerophilic yeasts and filamentous fungi from dried and semi-dry foods (Hocking and Pitt, 1980). Table 2.1 shows the compositions in g/L of the growth media used.

Table 2.1. Compositions and functions of PDA, MEA, NA and DG-18 in g/L.

COMPOSITION	PDA	MEA	DG-18	NA	FUNCTION
Agar	15	15	15	15	Solidifying agent
Dextrose	20	-	-	-	Energy source
Potato extract	4	-	-	-	Nutrition for fungi
Malt extract	-	30	-	-	C, N, protein sources
Yeast extract	-	-	-	2	Nutrition for bacteria
Peptone	-	5	5	5	N and vitamins sources
Glucose	-	-	10	-	Energy source
KH_2PO_4	-	-	1	-	Buffering agent
$MgSO_4$	-	-	0.5	-	Inorganic salt for fungi
Dichloran	-	-	0.002	-	Restricts colony size
Glycerol	-	-	220	-	C source
NaCl	-	-	-	5	For osmotic balance
Lab-Lemco[1]	-	-	-	1	Meat extract for bacteria
Cyclohexamide[2]	-	-	-	0.001	Antifungal compound
Chloramphenicol[3]	0.001	0.001	0.001	-	Antibacterial compound

[1] ***Laboratory of Liebig Extract of Meat Company***
[2] ***Added to the media post-autoclave***
[3] ***Added to the media pre-autoclave***

Cyclohexamide (IUPAC: 4-[(2*R*)-2-[(1*S*,3*S*,5*S*)-3,5-Dimethyl-2-oxocyclohexyl]-2-hydroxyethyl]piperidine-2,6-dione) was added (0.001 g/L) to the NA to inhibit fungal growth (Sigma-Aldrich, USA). This compound degrades with heat, and was added after sterilization of the medium by autoclaving. Chloramphenicol (IUPAC: 2,2-dichloro-N-[1,3-dihydroxy-1-(4-nitrophenyl)propan-2-yl]acetamide) was added (0.001 g/L) to the other media to inhibit bacterial growth (Fisher, UK). This compound does not degrade with heat, and was added before sterilization of the media by autoclaving. According to the manufacturer's instruction, to prepare the growth media, a specific amount of the media (PDA 39 g, MEA 50 g, NA 28 g, DG-18 31.5 g) was transferred separately into 1 L distilled water and autoclaved (Meadowrose, UK) at 121°C for 15 minutes at 15 psi. Autoclaved growth media were left to cool for 15 minutes and shaken well before being poured into 90 mm Ø Petri plates (≈15-20 mL per plate) and left to solidify. Solidified growth media were placed in sealed polyethylene bags and refrigerated in a cold room at 4°C for subsequent use.

2.2.4 Fungal isolation, enumeration, and identification

Isolation of mycobiota from maize kernels was performed on MEA and DG-18 by the direct-plating technique. Aseptically, five kernels of maize were directly placed onto the growth media at equal distance apart, and incubated at 25°C for seven days. All the treatments were carried out with three replicates per medium. At the end of the incubation period, all fungal colonies present were recorded and the isolation frequency (% IF) determined. Each fungal colony was also sub-cultured onto fresh PDA before being incubated at 25°C for another seven days to obtain axenic cultures (*i.e.*, pure and uncontaminated culture with only one species present). Axenic cultures on PDA were

used for fungal identification and were refrigerated in a cold room at 4°C until further examination.

Enumeration of mycobiota from maize kernels was performed in two ways; isolation frequency (% IF), and colony forming units (CFUs/g dry weight). To obtain isolation frequency, all fungal colonies growing from directly plated kernels on MEA and DG-18 were recorded and calculated according to Equation 2.2.

$$\textit{\% isolation frequency} = \frac{\textit{number of maize kernels colonized by a species}}{\textit{total number of maize kernels per plate}} \textit{ x 100}$$

Equation 2.2. Formula to calculate isolation frequency.

To calculate the fungal populations (CFUs), the serial dilution plating technique was performed on fresh plates of MEA and DG-18. Ten grams (10 g) of maize kernels were aseptically weighed and added to 90 mL sterilized 0.1% peptone water (*i.e.*, 1 g peptone in 1 L distilled water) in a Stomacher bag. The maize kernels were left to soak in the peptone water for an hour to soften before being homogenized by a Stomacher 400 (Seward, UK) for five minutes. The use of a Waring blender was avoided as the blending process introduces heat that may destroy fungal spores of several heat-sensitive species resulting in partial representation of the mycobiota diversity of maize kernels. The initial homogenization of maize kernels gave a 1:10 (10^{-1}) dilution. 10^{-2}, 10^{-3}, and 10^{-4} dilutions were achieved by three serial 10-fold dilutions with sterilized 0.1% peptone water. A 0.1 mL aliquot of the four dilutions was spread-plate on the different media (MEA, DG-18, NA) and evenly spread on the agar surface using a

sterile bent glass rod. All inoculated plates were incubated at 25°C for seven days. At the end of the incubation period, plates with 10-100 colonies were selected to calculate the fungal populations using Equation 2.3. To obtain the actual fungal load, the calculated CFUs were further adjusted based on the actual dry weight of the maize kernels after drying and reported as CFUs/g dry weight.

$$\textit{colony forming units} = \frac{\textit{number of colonies appeared}}{\textit{dilution of the plate counted x volume of aliquot}}$$

Equation 2.3. Formula to calculate colony forming units.

Identification of fungal isolates was carried out by means of morphology (*i.e.*, macro and micro). For macromorphology, colony forms, diameters, heights, textures and colours, sporulation density, growth rates, and reverse colours were recorded. For micromorphology, an optical microscope (Olympus, Japan) was used to observe spore forms and sizes, sporangiophores, as well as hyphal colours and sizes. An identification manual for food and indoor fungi (Samson *et al*., 2010) was used to identify the fungal isolates based on the morphological information recorded.

2.2.5 Isolation and molecular identification of the Malaysian strain of *Fusarium verticillioides* FV1

Isolation of *F. verticillioides* from Malaysian maize kernels was performed on MEA and DG-18 by the direct-plating technique as described in Subsection 2.2.4. Because the *Fusarium* strains were relatively slow growing (*i.e.*, reaching a hyphal diameter of only 35-55 mm in four days), cultivation of *F. verticillioides* was often unsuccessful on

MEA/PDA because they were overgrown by other fast-growing fungi (*i.e.*, *Rhizopus* spp., *Aspergillus* spp.). Therefore, surface sterilization of maize kernels was carried out with 5% (v/v) sodium hypochlorite (NaOCl) solution for five minutes to isolate internally present fungal species. These were plated on the growth media and incubated at 25°C for seven days. At the end of the incubation period, *F. verticillioides* colonies present were subcultured onto fresh PDA before being incubated at 25°C for another seven days to obtain axenic cultures. Axenic cultures of *F. verticillioides* on PDA were used for morphological and molecular identification. Morphological identification was performed as described in Subsection 2.2.4.

(a) DNA extraction

To identify *F. verticillioides* molecularly, DNA extraction was carried out by inoculating the strain onto the surface of MEA plates overlaid with a sterile sheet of cellophane. These were incubated at 25°C for five days because young cultures will release intracellular DNA more easily. On day five, the mycelial mat of *F. verticillioides* was harvested by removing it from the cellophane sheet, snap-freezing with liquid nitrogen, and stored at -80°C until further analysis. The use of cellophane sheet avoided contamination of DNA with media components.

The CTAB method was employed for the extraction of genomic DNA of *F. verticillioides* as described by Rodríguez *et al*. (2012) with minor modification. This method consisted of four major steps.

(i) Mechanical disruption of fungal mycelium

Fungal mycelium was ground to a fine powder in liquid nitrogen by using a pestle and mortar. Fine tissue powder was transferred to 2.0 mL safe-lock tubes (Eppendorf, Germany) and placed in an ice box to provide low temperature conditions for the DNA materials while liquid nitrogen was left to evaporate.

(ii) Cellular lysis by CTAB buffer

Five hundred microliter (500 µL) CTAB lysis buffer (Sigma-Aldrich, USA) and 10 µL proteinase K (10 mg/mL) were added to the Eppendorf tube containing the DNA materials, and the tube incubated at 65°C for one hour in a pre-heated water bath. The CTAB lysis buffer contained 5 g D-Sorbitol, 2 g N-lauroylsarcosine, 1.6 g CTAB, 9.4 g 1.4 M NaCl, 1.6 g 20 mM Na_2EDTA, 2 g PVPP in 0.1 M Tris-HCl pH 8.0. CTAB (cetyl-trimethyl ammonium bromide) is a lysis buffer/detergent (anion-binding reagent) that lysed cell wall and membrane systems to allow DNA out of the nucleus, and also precipitate negatively charged cellular proteins and polysaccharides. Adding NaCl to CTAB solution helps binding the proteins in cell lysate, thus separating the DNA from protein. Tris buffer (tris-hydroxymethyl-aminomethane) was used to maintain a stable pH because DNA is pH sensitive. EDTA (ethylene-diamine-tetra-acetic acid) is a chelating agent and has great affinity towards divalent metal ions (Ca^{2+}, Mn^{2+}, Mg^{2+}). Cellular content is rich with nucleases. After cellular lysis, DNA is exposed to DNAse in which Mg^{2+} is a cofactor. EDTA has four carboxyl groups (-COOH). In the alkaline condition of the buffer, EDTA becomes negatively charged and forms covalent bonds with Mg^{2+} preventing them from activating DNAse. As a result, DNAse is deactivated and will not degrade DNA. At the end of the incubation period, the tube was centrifuged

at 13,000 rpm (Zentrifugen, Germany) for five minutes at 4°C. The pellet was discarded, and the supernatant transferred into a new tube.

(iii) Phase separation by chloroform

Five hundred microliter (500 µL) chloroform was added to the supernatant before being vortexed, and centrifuged at 13,000 rpm for 20 minutes at 4°C. Chloroform ($CHCl_3$) which is an organic solvent separated the aqueous layer (upper; clear colour, contains hydrophilic DNA) from the organic layer (lower; bright pink contains cell wall, carbohydrates, lipids, proteins). The upper layer was transferred into another tube with 10 µL RNAse solution (10 mg/mL), and incubated for one hour at 37°C. At the end of the incubation period, 500 µL chloroform was added, and the tube was again vortexed and centrifuged at 13,000 rpm for five minutes at 4°C.

(iv) DNA precipitation

From previous step, DNA was now soluble in the buffer. To extract from the solution, the DNA was made insoluble by adding ethanol or isopropanol (isopropyl alcohol). Five hundred microliters (500 µL) absolute isopropanol was added to the tube, and incubated for 30 minutes at -20°C. When this was done, the DNA became visible in solution as a white thread-like substance. After incubation, the tube was centrifuged at maximum speed, for two minutes, at 4°C. The supernatant was discarded. The step was repeated again with 500 µL absolute ethanol. The supernatant was again discarded and the remaining ethanol was dried with blotting paper. When insoluble, DNA was unusable. After precipitation, the alcohol was removed as it inhibits DNA in PCR, and DNA was returned to a biological buffer to make it usable by re-suspending it in 50-100 µL TE

buffer at pH 8.0. To measure the concentration, yield and purity of the DNA materials extracted, conventional absorbance measurement was used, where nucleic acids have a maximum absorption at 260 nm, while other contaminants (*e.g.*, proteins, single stranded DNA, RNA) were absorbed at 280 nm. Absorbance was measured at 260 and 280 nm using the Picodrop with TE buffer as a blank. A software package, Picodrop Version 3.1.0.0 (Picodrop, UK), was linked to the pipette, and was used to automatically generate DNA concentrations, yield and quality. Equation 2.4 shows the formula to calculate the purity of nucleic acids (DNA/RNA) in the presence of contaminants. A ratio between 1.8 and 2.2 is indicative of highly purified DNA (Ahmad-Ganaie and Ali, 2014; Gallagher, 2001). DNA materials was later stored at -20°C for PCR amplification.

$$\textit{purity of nucleic acids DNA/RNA} = \frac{A[260]}{A[280]}$$

Equation 2.4. Formula to calculate the purity of nucleic acids DNA/RNA.

(b) PCR amplification

The extracted DNA material was further amplified through the polymerase chain reaction (PCR). In molecular identification of fungi, the internal transcribed spacer (ITS) region of nuclear DNA (nrDNA) is the preferred DNA barcoding marker both for the identification of single taxa and mixed environmental samples (Bellemain *et al.*, 2010) as it evolves the fastest as compared to the mitochondrial DNA (useful for Family identification) and the small-subunit nrDNA (useful for identification of distantly related microorganism; White *et al.*, 1990). To obtain a higher stringency (*i.e.*, sequence

amplification with near zero mismatches) and thus, higher specificity, two pairs of primers were used in the amplification of DNA material; ITS1-ITS2 and ITS3-ITS4. All primers were obtained commercially (Sigma-Aldrich, USA). The primer nucleotide sequences are shown in Table 2.2. The expected size for ITS1-2 pair was 283 kb and ITS-3-4 pair was 386 kb. To avoid contamination, DNA material, reaction mixture preparation, and the PCR procedure were performed in a designated area. Equipments/apparatus used were sterilized by UV irradiation, and solutions were sterilized by autoclaving except dNTPs, primers and *Taq* DNA Polymerase. A control reaction without the addition of DNA was included to confirm the absence of contamination. Table 2.3 shows the amounts of reagent for 50 µL reaction mixture per individual PCR tube. All reagents which were stored at -20°C were brought to room temperature and vortexed gently after thawing. PCR tubes were placed on ice. After the addition of all reagents, PCR tubes were vortexed gently, and placed in a thermal cycler (Techne, UK).

Table 2.2. Nucleotide sequences of ITS primers (adapted from White *et al.*, 1990).

FORWARD PRIMER	NUCLEOTIDE SEQUENCE	POSITION
ITS1	TCCGTAGGTGAACCTGCGG	1761-1779
ITS3	GCATCGATGAAGAACGCAGC	2024-2045
REVERSE PRIMER	**NUCLEOTIDE SEQUENCE**	**POSITION**
ITS2	GCTGCGTTCTTCATCGATGC	2024-2043
ITS4	TCCTCCGCTTATTGATATGC	2390-2409

PCR reaction conditions were set to 40 cycles which consisted of initialization step (94°C, five minutes), denaturation step (94°C, one minute), annealing step (50°C, one minute), extension/elongation step (72°C, two minutes), and final elongation (72°C, five minutes).

Table 2.3. Reagents amount for 50 µL reaction mixture.

REAGENT	AMOUNT PER SAMPLE (µL)
10X PCR buffer	5
dNTPs mix	1
Primer I (forward)	2
Primer II (reverse)	2
Taq Polymerase	0.5
$MgCl_2$	3
Extracted DNA sample	2
Sterilized deionized water	34.5

(c) Gel electrophoresis

To validate the success of the PCR amplification, the PCR product (amplicon) was run on agarose gel electrophoresis. One gram agarose powder in 50 mL tris acetate EDTA (TAE) 1X buffer (2% w/v) was heated in a microwave oven for one minute until it was dissolved completely before being loaded into small electrophoresis recipient (minigel, eight wells) and left to solidify with a comb fitted in to create wells. Two percent (2%) gel will show good resolution for small fragments (0.2–1 kb). TAE 1X buffer solution as running buffer was poured onto the solidified agarose gel to submerge the gel surface to 2-5 mm depth. To start the electrophoresis, a DNA ladder/molecular size marker

(0.15 – 2.2 kbp; Promega Biosciences, USA), loading buffer (dye; bromophenol blue), and PCR amplicons were prepared. Firstly, 2 µL DNA marker was mixed with 2 µL loading buffer, and this was loaded into the first well (left-most lane). Loading buffer gives colour (easy to monitor progress) and density (easy to load in wells) to the PCR amplicons. It is also negatively charged, and moves in the same direction as the DNA during electrophoresis. Two replicates (5 µL each) of PCR amplicons amplified by ITS1-ITS2 primers pair were loaded in the second and third lanes after being mixed with 2 µL loading buffer. The fourth lane was loaded without DNA sample (control). Fifth and sixth lanes were loaded with DNA samples amplified by ITS3-ITS4 primers pair. The seventh lane was loaded without DNA sample (control). Electrophoresis was run at 80 V. Electrophoresis was ended when the loading buffer has run 3/4 the length of the gel. Ethidium bromide was added onto the gel before the gel was viewed under UV with the aid of Genesnap image acquisition software version 7.09.02 (Syngene, UK).

When viewed under UV, successful PCR amplification produced a distinctive single band with length corresponding to that of the target DNA which can easily be compared against the DNA ladder. This indicates that a specific region of the DNA was successfully amplified without contamination. PCR amplicons was further sent for purification and sequencing (Macrogen, The Netherlands). DNA sequence obtained was analysed with Applied Biosystems Sequence Scanner version 1.0 and CLC Sequence Viewer version 6.6.1 before being aligned and compared by BLAST (Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov) to identify the strain.

2.2.6 Fumonisin production by the Malaysian strain of *Fusarium verticillioides* FV1 and other reference strains on a Fumonisin-inducing Medium (FIM)

Three strains of *F. verticillioides* namely *F. verticillioides* (FV1; isolated from Malaysian maize kernel), *F. verticillioides* MPVP 294 (reference strain; Formenti *et al.*, 2012; Etcheverry *et al.*, 2009) and *F. verticillioides* ITEM 1744 (reference strain; Lazzaro *et al.*, 2012a) were analysed for toxigenic potential by inoculating 100 µL spore suspension of the strain onto the fumonisin-inducing medium (FIM) according to López-Errasquín *et al*. (2007). The composition of FIM in g/L was malt extract (0.5), yeast extract (1), peptone (1), KH_2PO_4 (1), $MgSO_4 \cdot 7H_2O$ (0.3), KCl (0.3), $ZnSO_4 \cdot 7H2O$ (0.05), $CuSO_4 \cdot 5H_2O$ (0.01), fructose (20) and bacteriological agar (15). The inoculated growth medium was then incubated at 25°C. After 14 and 21 days, mycelial plugs (≈7 mm Ø) of the culture were taken from the centre of the plate (matured area). The mycelial plugs were analysed for fumonisins production according to the AOAC method (2002).

The AOAC Official Method (2001.04) involved transferring the mycelial plugs into a 2 mL safe-lock tube (Eppendorf, Germany) in triplicate and weighing them on an analytical balance (Ohaus, Switzerland) to an accuracy of ± 0.01 g. The weights were recorded. Next, 1,000 µL of extraction solvent (*i.e.*, methanol:acetonitrile:water, 25:25:50 v/v) was added to the tubes and the tubes left to extract for 30 minutes. After incubation, the tubes were vortexed vigorously, and centrifuged at 10,000 rpm with a micro-centrifuge (Biofuge, Germany) for five minutes. The supernatant was carefully filtered (nylon syringe filter, 0.22 µm aperture; Jaytee, UK) into a new tube. The filtrate

was evaporated to dryness at approximately 60°C, and the residue redissolved in 500 µL of HPLC-FLD mobile phase.

Separation and detection of FBs were performed by using a reversed phase-HPLC system joined to a fluorescence detector with pre-column derivatization of samples with OPA. The HPLC-FLD system was an Agilent 1200 series system (Agilent, UK) with a fluorescence detector (FLD G1321A), auto sampler (ALS G1329A), micro-oven (FC/ALS-therm G1330B), degasser (G1379B), bin pump (G1312A) and a C_{18} column (Cronus Nucleosil 100 C18, 4.6 x 150 mm, 5 µm; SMI-LabHut Ltd., UK) joined to a pre-column (security guard, 4 x 3 mm cartridge; Phenomenex, USA) for separation at 25°C. The mobile phase was methanol:0.1 M NaH_2PO_4 (77:23, v/v), adjusted to pH 3.35 using phosphoric acid (AOAC, 2001), filtered through a membrane (0.45 µm aperture), and pumped at 1 mL per minute. The chromatographic parameters were 335 nm for the excitation wavelength (λ_{ex}) and 440 nm for the emission wavelength (λ_{em}). The injection volume was 30 µL. Before injecting the standard/sample into HPLC system, 50 µL of standard/sample was derivatized with 100 µL OPA solution within one minute. OPA solution for derivatization of standards and samples was prepared by dissolving 40 mg *ortho*-phthaldialdehyde (Sigma-Aldrich, USA) in 1 mL absolute methanol before adding 5.0 mL 0.1 M disodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$) and 50 µL 2-mercaptoethanol (Sigma-Aldrich, USA) in capped amber vial (light sensitive). The chromatographic data (luminescence unit peak area) were integrated and calculated using a ChemStation (Agilent, UK) software programme.

A linear standard curve was constructed to transform luminescence unit (LU) peak areas obtained through HPLC analysis into actual concentration of fumonisins from the hyphal plug samples (μg/g). Analytical standard fumonisins mixture (50 μg/mL FB_1 + FB_2 in acetonitrile:water, 1:1 w/v) was purchased from Sigma-Aldrich, USA, and diluted to 0.1, 0.5, 2.5 and 5.0 μg/mL calibrant solutions. Five readings (n = 5) were recorded for each concentration of calibrant solutions (Σ = 20) intermittently throughout the sample analysis to check for reproducibility and reliability of quantification procedure. The chromatograms for the four calibrant solutions are shown in Appendix C. The linear standard curve was constructed by plotting the LU peak area of FBs versus concentrations of 0.1, 0.5, 2.5 and 5.0 μg/mL and is shown in Appendix D. The limit of detection (LOD) and limit of quantification (LOQ) were estimated by using the formulae; LOD = $3\sigma/s$ and LOQ = $10\sigma/s$, in which σ is standard deviation of y-intercepts of the FB_1 linear calibration curve, and s is the slope of the calibration curve (Medina and Magan, 2012).

2.2.7 Ecophysiological studies of the Malaysian strain of *Fusarium verticillioides* FV1 on milled-maize agar (MMA)

To examine the effects of ecophysiological conditions (*i.e.*, a_w × temp.) on growth rates and fumonisin production by FV1 *in vitro*, a milled-maize agar (MMA) was used. MMA was formulated to mimic the nutritional composition of natural maize *in vitro*. The MMA was prepared by grinding 50 g dent maize kernels before sifting the powder with a stainless steel test sieve (200 mm Ø, 0.5 mm aperture; Endecotts, UK) to obtain uniform and fine maize powder. Thirty gram (30 g) fine maize powder and 15 g technical agar were added to 1 L distilled water. This was modified by the addition of

the non-ionic solute glycerol to obtain 0.937 (0.93 a_w), 0.955 (0.95 a_w), and 0.982 (0.98 a_w) as detailed by Dallyn and Fox (1980) by adding 32.2, 23, and 9.2 g glycerol per 100 mL distilled water respectively, and autoclaved (Meadowrose, UK) at 121°C for 15 minutes at 15 psi. Autoclaved MMA was left to cool for 15 minutes before being poured into 90 mm Ø Petri plates (≈15-20 mL per plate) and left to solidify. Solidified MMA plates were placed in sealed polyethylene bags and refrigerated in a cold room at 4°C until inoculation with FV1.

To inoculate the MMA plates, germinating spores of FV1 was obtained by decanting 15 mL sterilized 0.1% Tween-80 solution (v/v) onto a 10-day axenic culture of the fungus on MEA. Sterilized bent glass rod was used to scrape the culture surface to better dislodge the fungal spores during which Tween-80 acted as a surfactant. The spore suspension was later transferred into a sterilized Universal bottle. A Helber haemocytometer (depth 0.02 mm; Marienfeld, Germany) was used to estimate the concentration of the spore suspension. Steps to calculate spores from the square-grids of the haemocytometer are described in Appendix E. FV1 spores were adjusted to $\approx 10^6$ spores/mL before aseptically aliquotting100 µL onto the MMA plates and spreading it evenly by using a sterilized bent glass rod. Each of the a_w-modified-MMA (*i.e.*, 0.93, 0.95, 0.98 a_w) was inoculated in triplicates for each of the temperature (*i.e.*, 20, 25, 30°C). Incubation period was set to ten days by placing the inoculated MMA plates in separate sealed containers each supplied with a jar of corresponding a_w to maintain the condition throughout the incubation period. The radial hyphal extension (mm) of FV1 colonies was measured after two, four, six, eight and ten days in two directions at 90° angle to each other. At the end of this, the growth rates of FV1 were calculated by

taking the slope (*m*, from $y = mx + c$) from a linear regression of radial hyphal extension (mm) on the *x*-axis against the incubation period (time in days) on the *y*-axis. Mycelial plugs (≈7 mm Ø) from the MMA plates were also taken, weighed, solvent-extracted, and analysed for FB_1 production by HPLC-FLD according to procedures described in Subsection 2.2.6.

2.2.8 Statistical analysis

All experiments were carried out with three replicates per treatment. Measurements were then averaged and presented as mean ± SE (standard error). Normal distribution of datasets was checked by the Kolmogorov-Smirnov normality test. Analysis of Variance (ANOVA) was applied on normally distributed datasets to analyse the variation between and within group means with 95% confidence interval. $p < 0.05$ was accepted as significantly different. Fisher's Least Significant Difference (LSD) with $\alpha = 0.05$ was applied to compare significance of differences between means of treatments using the statistical software Minitab® version 14.0 (Minitab Inc.; Pennsylvania, USA).

Figure 2.2 summarizes the key experimental steps carried out in this Chapter.

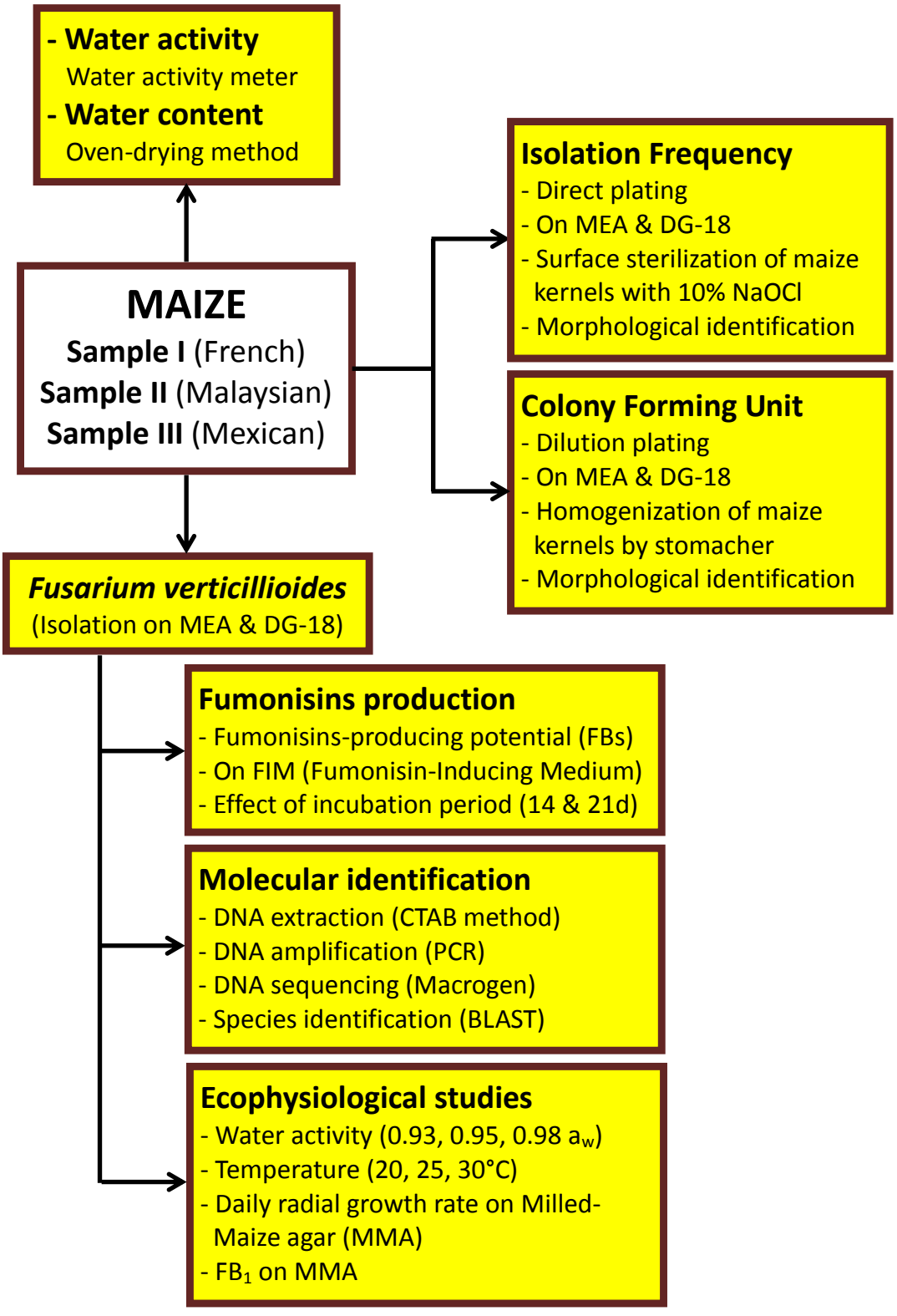

Figure 2.2. Key experimental steps carried out in Chapter 2 "Biodiversity of mycobiota in maize samples from different regions".

2.3 RESULTS

2.3.1 Water content and water activity of maize kernel samples

Figure 2.3 shows the water content and a_w of maize samples from different regions. This ranged from 4.7 to 36.8% and 0.400 to 0.970 a_w respectively. Sample 2 had the highest water content (36.8 ± 2.3%) and a_w (0.970 ± 0.001). The sample from Malaysia was very moist and had to be stored at 4°C to ensure preservation without fungal spoilage.

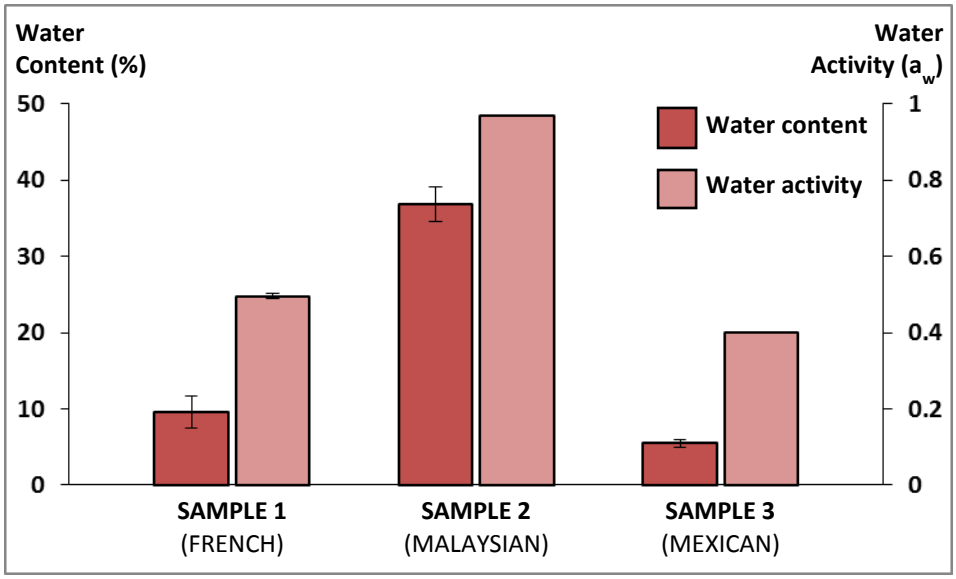

Figure 2.3. The water content (%) and water activity (a_w) of maize samples from climatically and culturally-different regions. Data are means of triplicates (*n* = 3) with bars indicating standard error.

2.3.2 Isolation frequency and fungal populations in maize samples

(a) Isolation frequency of fungi from maize samples

Figures 2.4 and 2.5 compare the predominant fungal genera and species isolated from the French and Mexican maize samples respectively when they were either surface sterilized or plated without surface sterilization. These show that there was a higher

diversity on unsterilized maize kernels than that of surface sterilized. On DG-18 however, the predominant genera were restricted to a few with *A. flavus*, *F. verticillioide*s, *Penicillium* sp. or *Eurotium* sp. In the case of the Malaysian maize samples, because of the high a_w level, *F. verticillioides* was isolated from all the kernels (100% IF), where surface sterilized or not. Thus, no figure is presented for this data set.

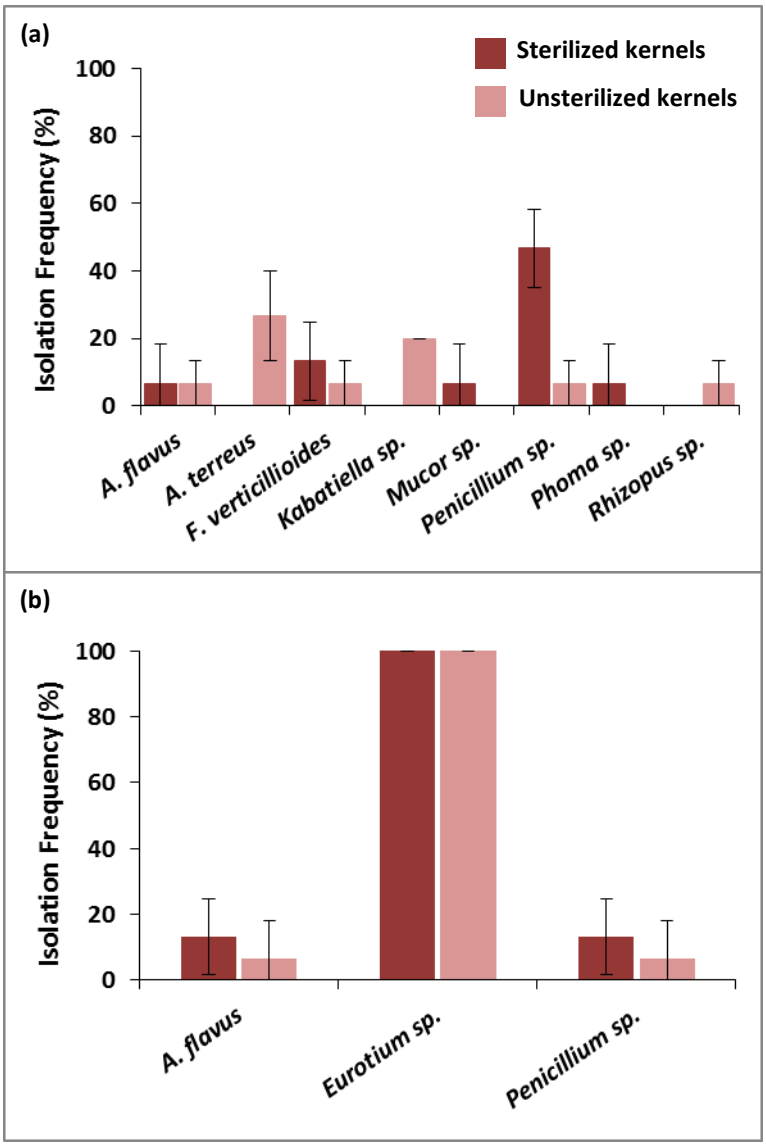

Figure 2.4. Isolation frequencies (% IF) of predominant fungi isolated from sterilized and unsterilized sample 1 (French) maize kernels on (a) MEA and (b) DG-18 after seven days incubation at 25°C. Data are means of triplicates (n = 3) with bars indicating standard error.

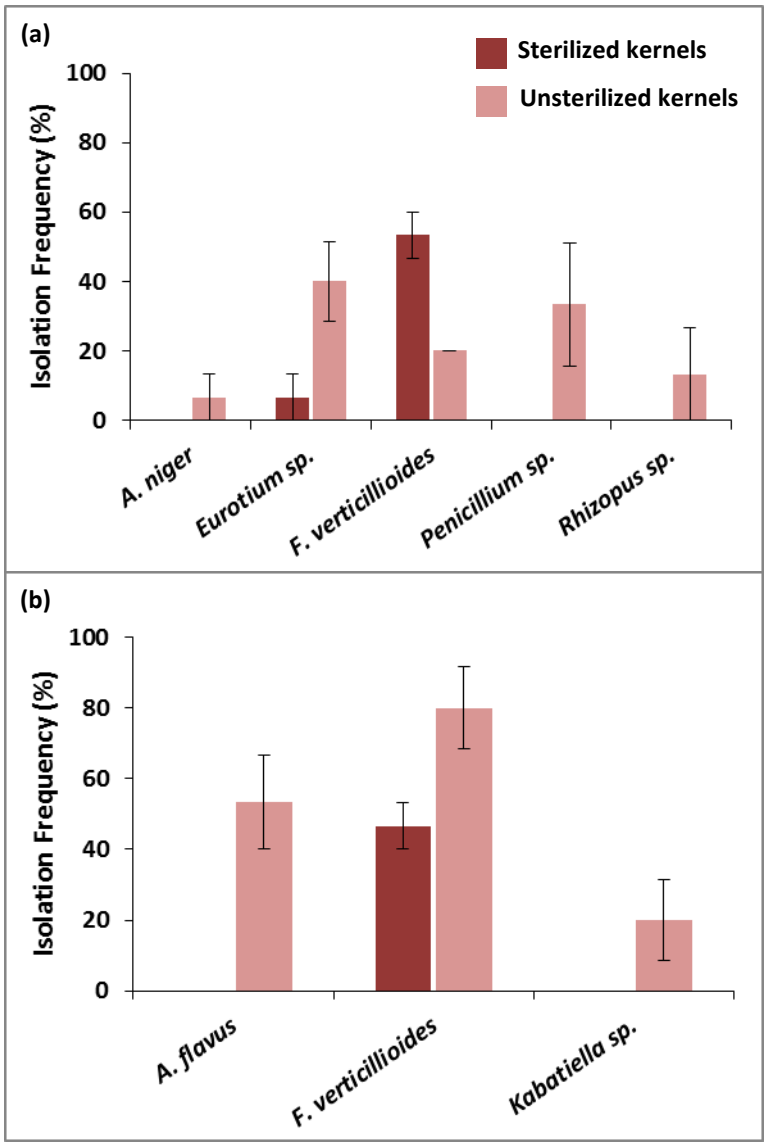

Figure 2.5. Isolation frequencies (% IF) of predominant fungi isolated from sterilized and unsterilized sample 3 (Mexican) maize kernels on (a) MEA and (b) DG-18 after seven days incubation at 25°C. Data are means of triplicates (n = 3) with bars indicating standard error.

(b) Fungal populations isolated from maize samples

In terms of total fungal populations isolated from the maize samples on both MEA and DG-18, a wide range of mycobiota was present: 10^3 CFUs/g dry weight from the French samples, 10^6 CFUs/g dry weight from Malaysian samples, and 10^2 CFUs/g dry weight

from the Mexican samples on both growth media (Figure 2.6). Positive correlations were observed between water content and total fungal populations (Pearson's *r* MEA = 0.9682; *r* DG-18 = 0.9776), and between a_w and total fungal populations (Pearson's *r* MEA = 0.9822; *r* DG-18 = 0.9870).

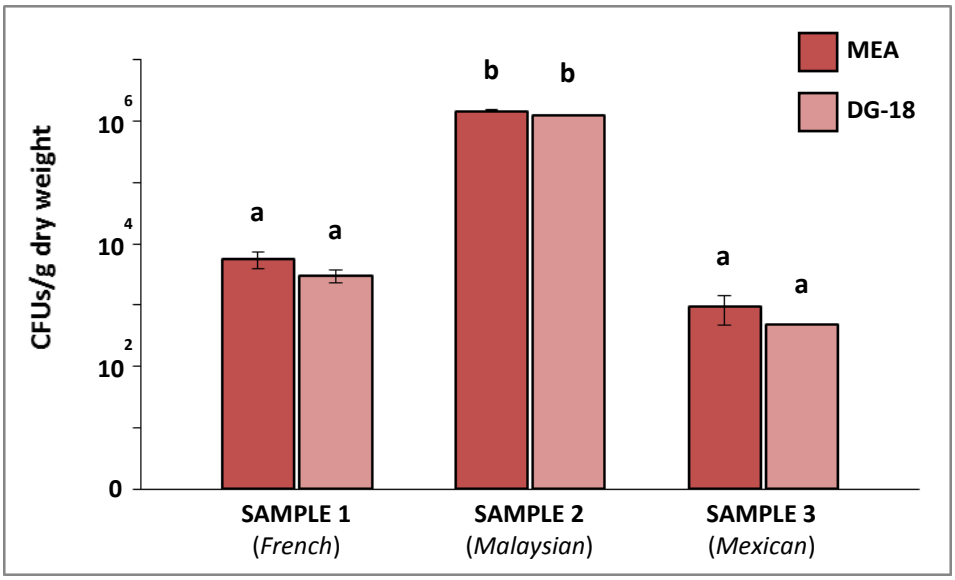

Figure 2.6. Total fungal populations (CFUs/g dry weight) isolated from maize samples plated on MEA and DG-18. Data are means of triplicates (n = 3) with bars indicating standard error. Different letters indicate significant difference ($p < 0.05$) by Fisher's Least Significant Difference (LSD).

2.3.3 Isolation and molecular identification of *Fusarium verticillioides* FV1

A strain of *Fusarium* sp. was isolated directly from the Malaysian maize in an almost pure culture (Figure 2.7a). This appeared to have the morphological characteristics of *F. verticillioides* such as producing salmon-coloured colonies on DG-18, dense, whitish and delicately floccose (cottony) aerial mycelia with a powdery appearance due to presence of microconidia (Samson *et al.*, 2010). When subcultured onto MEA, the

resulting pure culture exhibited lilac-coloured colony with whitish to vinaceous cottony aerial mycelia (Figure 2.7b).

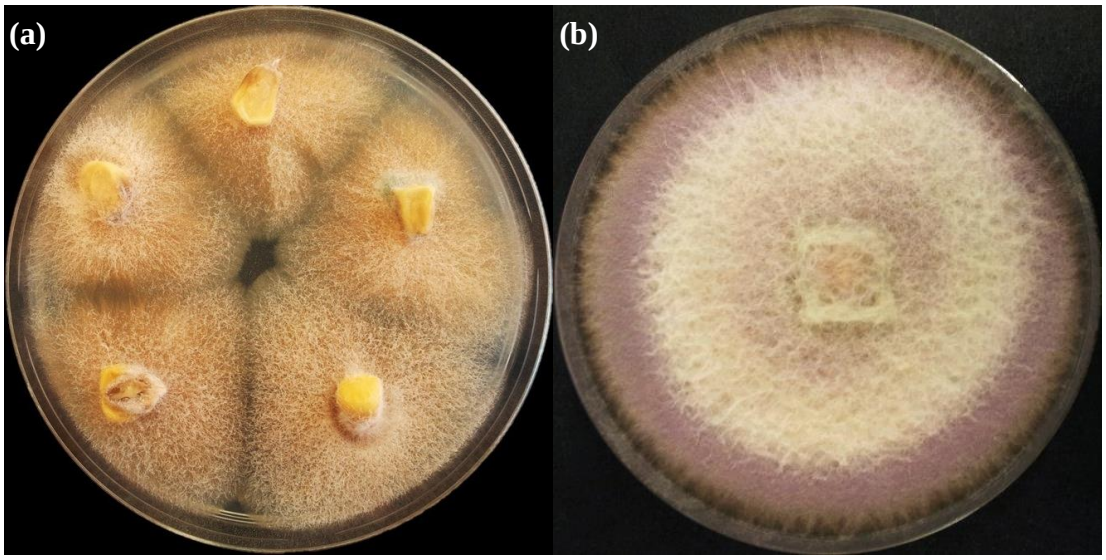

Figure 2.7. Cultural appearances of Malaysian strain of *Fusarium* sp. from (a) the isolation medium (DG-18) with salmon-coloured colonies, and (b) the cultivation medium (MEA) with lilac-coloured pure colony, after ten days incubation at 25°C.

To further validate the identity of this isolate, molecular identification (*i.e.*, DNA extraction, PCR with ITS primers, DNA purification and sequencing) was carried out in which the DNA sequence of the fungal isolate matched 100% with that of an existing *Fusarium verticillioides* strain GNU-F11 (GenBank accession number KC752592.1) in the BLAST database (Figure 2.8). This isolate was henceforth called *F. verticillioides* FV1 (FV1). The electrophoresis gel is shown in Appendix F.

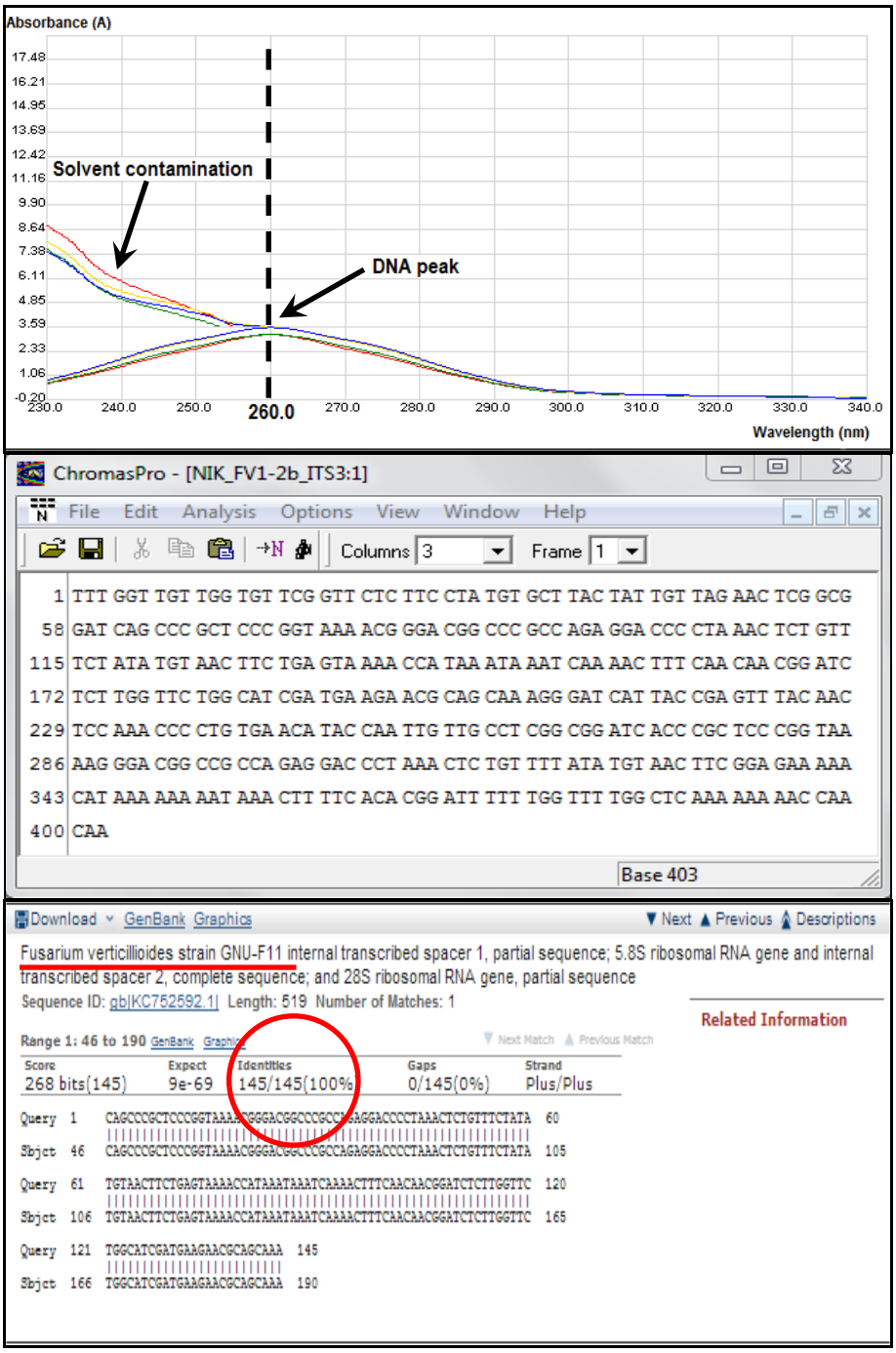

Figure 2.8. Major steps in molecular identification of the Malaysian strain of *Fusarium* sp.
(top) DNA extraction of *Fusarium* sp. showing DNA yield and quality
(middle) Gene sequencing of *Fusarium* sp. from ITS amplification
(bottom) Validation of the gene sequences against that of an existing *F. verticillioides* sequence in the BLAST database

2.3.4 Comparison of fumonisin production by *Fusarium verticillioides* FV1 and two reference strains on a Fumonisin-inducing Medium (FIM)

The production of FBs (FB_1, FB_2) by FV1 and two reference strains (*F. verticillioides* MPVP 294, *F. verticillioides* ITEM 1744) after 14 and 21 days growth is shown in Figure 2.9. It was found that FV1 produced much higher amounts of FB_1 and FB_2 than the two reference strains. In addition, between day 14 and 21, there was a significant reduction in the amounts of FB_1 and FB_2 present in extracts of the cultures.

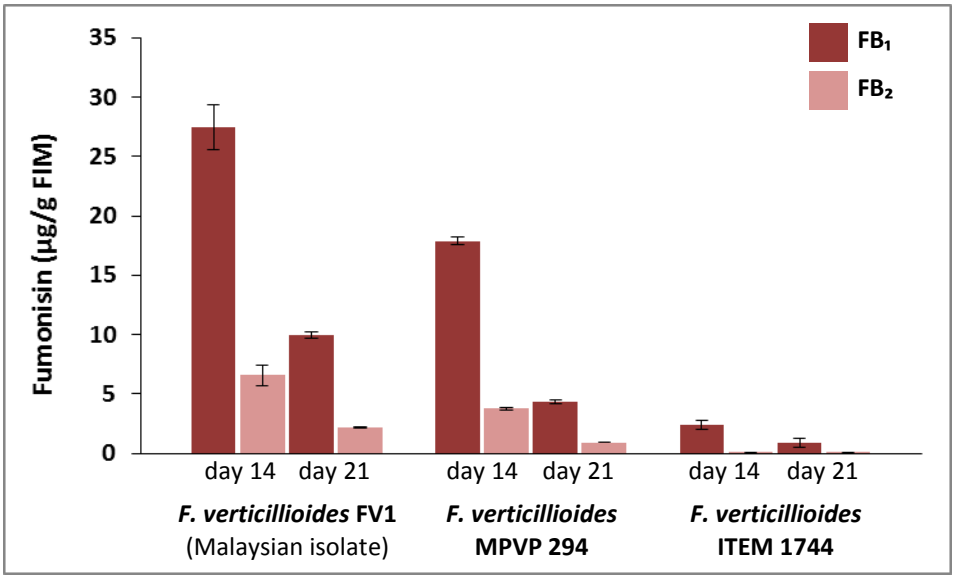

Figure 2.9. Comparison of fumonisin production (FB_1, FB_2) by different strains of *F. verticillioides* on a fumonisin-inducing medium (FIM) after 14 and 21 days incubation at 25°C. Data are means of triplicates (n = 3) with bars indicating standard error.

2.3.5 Effect of water activity × temperature on growth and fumonisin B_1 production by *Fusarium verticillioides* FV1 on milled-maize agar (MMA)

The effect of different a_w levels and temperatures on radial growth of FV1 on MMA after 10 days incubation is shown in Figure 2.10. There was a significant effect of temperature ($p < 0.05$) especially between 20 and 25°C, with less difference between 25 and 30°C, irrespective of a_w. The growth rate of FV1 increased from 0.93 to 0.98 a_w with significantly better growth at 25-30°C at all three a_w levels.

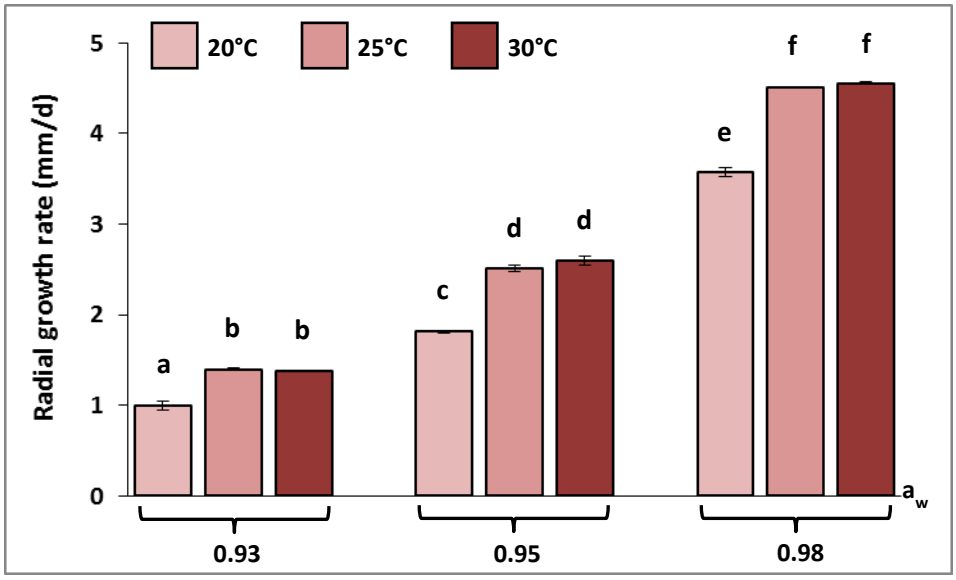

Figure 2.10. Radial growth rates (mm/d) of *Fusarium verticillioides* FV1 on milled-maize agar (MMA) at 0.93, 0.95 and 0.98 a_w, and 20, 25, and 30°C after 10 days incubation. Data are means of triplicates ($n = 3$) with bars indicating standard error. Different letters indicate significant difference ($p < 0.05$) by Fisher's Least Significant Difference (LSD).

The effects of a_w × temperature changes on FB_1 production by FV1 on MMA after 10 days incubation is shown in Figure 2.11. As for relative growth rates, the higher FB_1 production was at 25 and 30°C, irrespective of the a_w level. In addition, at both 0.95 and 0.98 a_w, there was a marked increase in the levels of FB_1 at 25°C when compared to 30°C. However, at 0.93 a_w there were similar levels of FB_1 at both 25 and 30°C. Overall, the highest concentration of FB_1 production by FV1 was at 0.95 a_w at all temperatures tested.

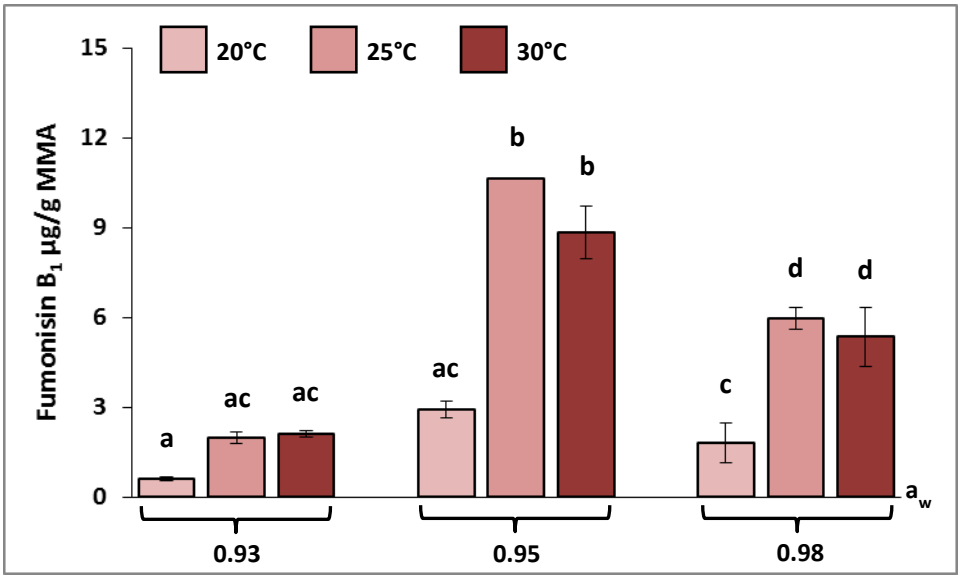

Figure 2.11. Fumonisin B_1 production (FB_1; µg/g MMA) by *Fusarium verticillioides* FV1 on milled-maize agar (MMA) at 0.93, 0.95 and 0.98 a_w, and 20, 25, and 30°C after 10 days incubation. Data are means of triplicates (n = 3) with bars indicating standard error. Different letters indicate significant difference (p < 0.05) by Fisher's Least Significant Difference (LSD).

2.4 DISCUSSION

2.4.1 Biodiversity of mycobiota in maize samples from different regions

The maize kernels were colonized by many fungal strains whose occurrence in terms of isolation frequency (% IF) varied with water content and a_w of samples and the type of isolation medium used (*i.e.*, DG-18, MEA), irrespective of whether they were surface sterilized or not. Spoilage fungi such as *Aspergillus*, *Penicillium* and *Fusarium* found in the present study were also isolated previously in maize (Gonzaslez *et al*., 2003; Kpodo *et al*., 2000; Marín *et al.*, 1998; Pitt *et al.*, 1994). This is because maize represents a very good nutritional substrate for fungal colonization (Kumar *et al*., 2008). The isolation of almost a pure culture of *F. verticillioides* from the Malaysian samples reflects the very high a_w level of these samples and the fact that they had not been effectively dried after harvest. This will have predisposed them to colonization by Fusaria which grow well at 0.98 a_w over a range of warmer temperatures (25-35°C). Furthermore, in other region (*e.g.* Brazil), Orsi *et al.* (2000) also found *F. verticillioides* to be predominantly isolated from freshly harvested and stored maize. The presence of *Penicillium* spp., *Kabatiella* sp., *Phoma* sp., and the zygomycetous *Rhizopus* spp. and *Mucor* spp. at varying isolation frequencies is probably because they are all soil- or air-borne and thus likely to result in surface contaminants of cereals (Samson *et al*., 2010).

Because MEA is a common cultivation medium, it gave slightly higher isolation of genera/species (eight from French samples, five from Mexican samples) than that isolated on DG-18 (three from both samples). The latter medium gives complimentary information and supports growth of only selected xerotolerant/xerophilic yeasts and

moulds from intermediate moisture foods (Hocking and Pitt, 1980). The immersion of maize kernels in 5% (v/v) sodium hypochlorite (NaOCl) solution for five minutes has a variation in effects (increase, decrease) on isolation frequencies of fungal isolates. This variation might be attributed to the composition of internal fungi within the maize kernels which were not affected by the surface sterilization (Ayalew, 2010). Therefore, no pattern could be established on the effect of surface sterilization on isolation frequencies of fungal isolates. In terms of total viable and cultivable fungal populations isolated from the maize kernel samples, there was a direct correlation between a_w of maize samples and the fungal loads.

On the whole, the mycobiota profile of maize kernel samples in terms of occurrence and abundance of total fungal populations provides information on the structure of non-mycotoxigenic and mycotoxigenic fungi occupying the maize kernel niche. High incidence of mycotoxigenic *A. flavus* and *F. verticillioides* reflects the mycotoxins they are able to produce on maize; aflatoxins and fumonisins respectively (Giorni *et al.*, 2011; Magan *et al.*, 2002). While there are no reports so far on mycotoxin production by the other fungal contaminants, several isolates from maize kernels in the present work are known to produce active secondary metabolites. Examples of such metabolites are diaporthinic acid and tenuazonic acid (acutely toxic in mammals) produced by *Phoma* spp. which colonize a wide range of food commodities (Sørensen *et al.*, 2010), and neoechinulin and cladosporin (respiratory allergens) produced by *Eurotium* spp. (Slack *et al.*, 2009).

This study only examined a limited number of samples in terms of mycobiota, so it only provides a snapshot of possible contamination of different maize kernels with fungi. This is because the main aim of the present Chapter was to isolate potential antagonists which could effectively compete and act as potential biocontrol agents of *F. verticillioides* and fumonisin production.

2.4.2 Effect of interacting ecophysiological factors on growth and fumonisin B_1 production by *Fusarium verticillioides* FV1 on milled-maize agar (MMA)

This study showed that the growth of the Malaysian strain FV1 responded to interacting a_w × temperature conditions in a similar way to other strains examined in Europe (Medina *et al.*, 2013; Marín *et al.*, 2004). Radial growth rates at 25 and 30°C were very similar irrespective of a_w level and suggests that FV1 can grow over a wide range of temperatures. Based on the a_w levels tested in this study, 0.98 a_w was optimum for growth of this strain. This is similar to what has been observed previously by Marín *et al.* (1999) that 0.98 a_w and 30°C were optimum for growth of strains isolated from Spanish maize. In these latter studies, 20 and 30°C were tested and suggested significantly slower growth rates at the lower temperature, irrespective of a_w level. Previously, initial germination rates of *F. verticillioides* strains were reported to be optimum at 25-37°C at >0.96 a_w but changed to 30°C at lower a_w levels (Marín *et al.*, 1996). The overall a_w × temperature profile for FV1 needs to be compared with the range of conditions previously suggested for *Fusarium* species generally, where the temperature range was suggested to be 2-37°C with an a_w range of >0.87-0.995 a_w (Lacey, 1989).

A relatively wide range of a_w for *F. verticillioides* growth might explain its ability to exist in dual-nature in maize (Bacon *et al*., 2008) in which it can exist as an endophyte in soil and root systems as well as a pathogen that is present in almost all parts of maize plant and able to inflict severe diseases in those parts (*e.g.*, seed rot, stalk rot, kernel rot). Battilani *et al*. (2011) have also demonstrated that the a_w range during silking is critical in determining infection and fumonisin contamination.

A_w × temperature also impacted on FB_1 production. Optimum FB_1 was at 25°C and 0.95 a_w. There was a significant decrease in FB_1 production when a_w was increased to 0.98 a_w. These findings fit into the general profile for FB_1 production by Spanish strains of *F. verticillioides* in boundary modelling of FB_1 production by *Fusarium* section Liseola species (Marín *et al*., 2004; 1999). The difference in temperature range over which FV1 grew best (25-30°C) and produced FB_1 (25°C) in the present study may be explained by the fact that the temperature range for FB_1 production is slightly narrower than that required for germination and growth (Figure 2.12).

2.5 CONCLUSIONS

Overall, biodiversity findings obtained in the present study are in agreement with relevant published data. A positive correlation was observed between water content and water activity of maize kernel samples which in turn significantly affected the abundance of mycobiota. Malaysian maize kernel samples being the moistest gave 100% *Fusarium* sp. isolates. All maize kernel samples exhibited occurrence of common cereal fungi (*Aspergillus* spp., *Fusarium* spp., *Penicillium* spp.) irrespective of different growth media and sterilization treatments employed. Molecular identification of

Fusarium sp. isolated from Malaysian maize kernels confirmed the morphological identification as being *Fusarium verticillioides*. Among the three *F. verticillioides* strains, the Malaysian strain produced the highest fumonisins on fumonisin-inducing medium. Therefore, it was coded as FV1, and used in subsequent experiments. In ecophysiological studies, water activity and temperature were found to be the major factors affecting the growth and FB_1 productions by *F. verticillioides* FV1. 0.98 a_w was optimum for growth while 0.95 a_w was optimum for FB_1 production at all temperatures tested. 25-30°C was optimum for growth, while 25°C was optimum for FB_1 production at all a_w tested.

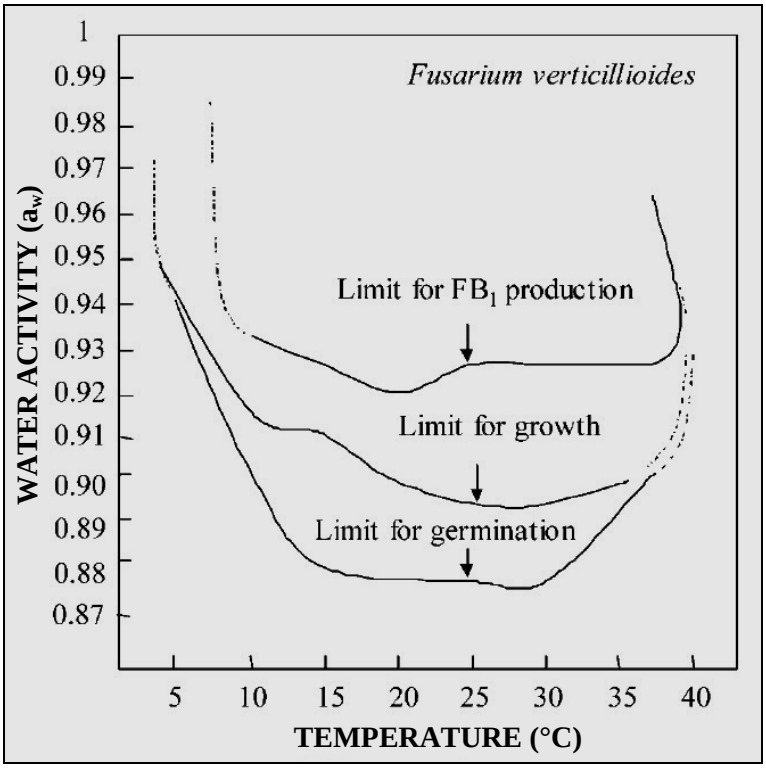

Figure 2.12. Isopleths showing the combined values of water activity and temperature on the boundary limits for FB_1 production (1 mg/g), growth (0.1 mm/d), and germination of *Fusarium verticillioides* (10% conidia). An isopleth is a line drawn through all points of equal value for several measurable parameters (adapted from Marín *et al.*, 2004).

CHAPTER 3

Efficacy of potential biocontrol agents for control of Fusarium verticillioides FV1 and fumonisin B_1 under different inoculum ratios and water activities on milled-maize agar and stored maize kernels

3.1 INTRODUCTION

The development of pre- and post-harvest control measures for mycotoxin contamination has received significant attention (Magan and Aldred, 2007a). For animal feed, often aliphatic acid-based preservatives have been used to try and minimize fungal spoilage and mycotoxin contamination during storage. The application of biological control agents (BCAs) using indigenous microbial inoculants is being increasingly recognized as a potential ecological approach to minimize pesticide inputs into the cultivation system (Köhl *et al.*, 2011; Xu *et al.*, 2011). Recently, Formenti *et al.* (2012) compared the efficacy of fungicides and a commercial BCA (Serenade; *Bacillus subtilis* strain QST713) under different water activity (a_w) regimes. They found that some fungicides and Serenade effectively controlled both *F. verticillioides* growth and FB_1+FB_2 production *in vitro*. Presently, three approaches have been used to try and address the endophytic and pathogenic phases of pathogens such as *F. verticillioides*. Seed-based applications with BCAs have been used to try and control the endophytic systemic colonization of seedlings of maize (Terzi *et al.*, 2014; Yuttavanichakul *et al.*, 2012); spray inoculations during silking with bacteria or yeasts has been examined to control mycotoxin contamination, especially by aflatoxins (Dorner and Cole, 2002; Oliveira *et al.*, 2014); and the application of non-toxigenic strains (*e.g.*, *A. flavus*) to soil and crop residue to reduce inoculum levels (Mauro *et al.*, 2014; Cotty *et al.*, 2008;

Cotty, 2006). There is interest in identifying appropriate BCAs which can compete effectively with *F. verticillioides* in the maize ecosystem and minimize fumonisin contamination.

Therefore, the objectives of this Chapter were:

(a) To isolate indigenous bacteria and yeasts from Malaysian maize kernels as potential biocontrol candidates against *F. verticillioides* FV1

(b) To screen the isolated candidates as well as to test existing biocontrol candidates for antagonism against *F. verticillioides* FV1 *in vitro* in terms of interaction scores, daily growth rate (mm/day) and colony development (cm^2)

(c) To examine the best potential biological control agents (BCAs) by using different ratios of pathogen:antagonist (100:0; 25:75; 50:50; 25:75; 0:100) on milled-maize agar (*in vitro*) and on stored maize kernels (*in vivo*) under different a_w conditions on relative control of fumonisin B_1 (FB_1) production

3.2 MATERIALS AND METHODS

3.2.1 Microorganisms

A fumonisin-producing strain of *Fusarium verticillioides* FV1 which was isolated from Malaysian maize kernels and identified morphologically and molecularly as described in Subsection 2.2.5 was used. Three biocontrol agents previously used to antagonize other fungal pathogens were obtained externally; *Clonostachys rosea* 016 by courtesy of Dr. Jürgen Köhl of Wageningen University and Research Centre, The Netherlands (Palazzini *et al.*, 2013); *Streptomyces* sp. AS1 by courtesy of Dr. Yousef Sultan of National Research Centre for Food Research, Egypt (Sultan and Magan, 2011); and *Enterobacter hormaechei* by courtesy of Prof. Miriam Etcheverry of National University of Río Cuarto, Argentina (Pereira *et al.*, 2011; 2010).

3.2.2 Isolation and identification of indigenous bacteria and yeasts from maize kernels

Isolation of indigenous bacteria and yeasts from maize kernels was performed on Nutrient agar (NA) and Potato Dextrose agar (PDA) respectively according to procedures described in Subsections 2.2.3 (preparation of growth media for microbial cultivation), and 2.2.4 (plating techniques). NA was supplemented with the antifungal compound cyclohexamide and PDA was supplemented with the antibacterial compound chloramphenicol. Inoculated plates were incubated at 25°C in triplicate for seven days. At the end of incubation period, bacterial and yeasts colonies grown on the agar were sub-cultured onto fresh NA and PDA respectively and incubated at 25°C for another seven days to obtain axenic cultures (*i.e.*, pure and uncontaminated culture with only

one species present). At the end of the incubation period, axenic cultures were refrigerated in a cold room at 4°C until further analysis. To identify bacterial isolates, Gram-staining was performed (Moyes *et al.*, 2009). First, a drop of distilled water was pipetted onto a clean slide before placing a loopful of bacterial culture on the water droplet. The slide was then quickly passed over a burning flame to heat-fix the bacterial cells. Heat-fixing killed and affixed the bacteria to the slide and avoided removal during the washing step. Next, a primary stain (crystal violet) was pipetted onto the heat-fixed smear before immediately adding iodine (mordant) to enhance crystal violet binding to the cell. After this, alcohol was applied in excess to decolorize the primary stain. Finally, the counterstain (red safranin) was applied to the bacterial smear. After 30 seconds, the slide was rinsed with distilled water, and excess liquid removed. The slide was observed under an optical microscope (Olympus, Japan). Immersion oil was added between the slide and the lense (100 x magnifications). Gram-positive bacteria appeared purple, and Gram-negative bacteria appeared red.

3.2.3 Antagonism of biocontrol candidates against *Fusarium verticillioides* FV1 in dual-culture assays

The dual-culture assay was adapted from Magan and Lacey (1984) to observe the interaction of two strains inoculated on the same culture plate (co-cultivation). The dual-culture assays of FV1 and potential antagonists were done on PDA for fungal candidates and on ½ NA for bacterial candidates. A hyphal plug of FV1 (≈ 5 mm x 5 mm dimensions) was inoculated approximately 30 mm from the plate wall and approximately 30 mm from the antagonist on both PDA and ½ NA (Figure 3.1). Point inoculation (hyphal plug) was preferred for fungal candidates (filamentous) while streak

inoculation was preferred for bacterial and yeast candidates. A plate inoculated with only the hyphal plug of FV1 on both PDA and ½ NA without the inoculation of any potential antagonist served as negative controls. The assay was carried out with three replicates per pathogen-antagonist pair and incubated at 25°C for seven days. At the end of incubation period, the types of interactions were scored as mutual intermingling (1/1); mutual antagonism upon contact (2/2); mutual antagonism at a distance (3/3); dominance of one species upon contact (4/0); dominance of one species at a distance (5/0). Diametric hyphal extension (mm) of FV1 on the dual-culture plates was measured at day 1, 3, 5, and 7 in two directions at right angles to each other (perpendicular). Diametric readings from replicates were averaged and divided by two to obtain radial data. Growth rates of FV1 were obtained by plotting the radial data on the *y*-axis against day of incubation on the *x*-axis. The slope (*m*) from the linear regression curve ($y = mx + c$) was used to calculate the daily radial growth rate (mm/day) of FV1. Colony development of FV1 (cm^2) was measured by fitting its colony radius (r) at day seven into the $\pi \cdot r^2$ formula in which π is 22/7.

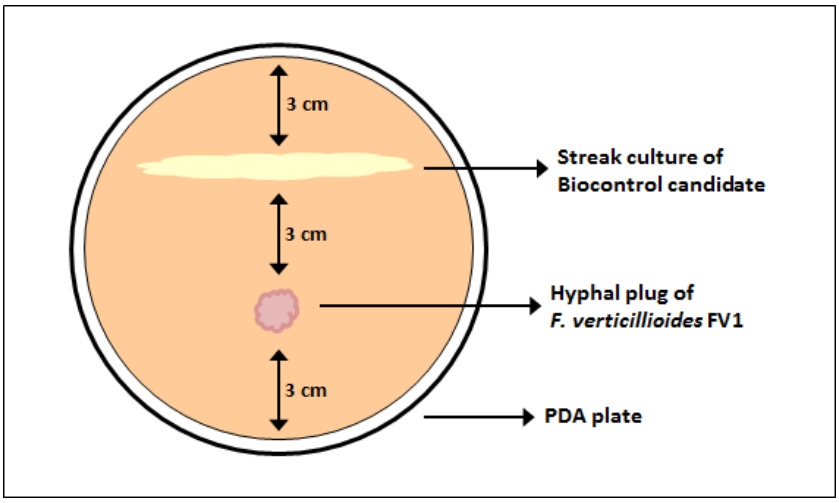

Figure 3.1. Schematic diagram of point inoculation for *Fusarium verticillioides* FV1 and streak inoculation for biocontrol candidates in dual-culture assays.

3.2.4 Growth media for pathogen:antagonist co-cultivation on maize agar and maize kernels

For pathogen:antagonist co-cultivation *in vitro*, a maize-based synthetic growth medium (3% milled-maize agar; MMA) was used. The MMA was prepared by grinding 50 g dent maize kernels before sifting the powder with a stainless steel test sieve (200 mm diameter, 0.5 mm aperture; Endecotts, UK) to obtain a uniform and fine maize powder. Thirty gram (30 g) fine maize powder and 15 g technical agar were added to 1 L distilled water. This was modified by the addition of the non-ionic solute glycerol to obtain 0.955 (0.95 a_w) and 0.982 (0.98 a_w) as detailed by Dallyn and Fox (1980) by adding 23 and 9.2 g glycerol per 100 mL distilled water respectively, and autoclaved (Meadowrose, UK) at 121°C for 15 minutes at 15 psi. Autoclaved MMA was left to cool for 15 minutes before being poured into 90 mm Ø Petri plates (≈15-20 mL per plate) and left to solidify. Solidified MMA plates were placed in sealed polyethylene bags and refrigerated in a cold room at 4°C for subsequent pathogen:antagonist co-cultivation *in vitro*.

For pathogen:antagonist co-cultivation *in vivo*, dry gamma-irradiated maize kernels were used. Dry maize kernels were commercially obtained from Bedfordshire, and irradiated at 12 kGy gamma irradiation (Isotron Plc; Swindon, UK) before being stored at 4°C. At this irradiation dose, the kernels were decontaminated from microbial propagules but had retained germinative capacity. To modify the a_w of the maize kernels, a moisture adsorption curve was constructed by weighing and transferring 5 g maize sub-samples into 11 dry 25 mL Universal glass bottles. The maize kernels were then hydrated by adding water into the bottles (0.0, 0.5, 1.0, 1.5, 2.0, 2.5., 3.0, 3.5, 4.0,

4.5 and 5.0 mL), and mixed thoroughly. All bottles containing hydrated maize kernels were sealed and refrigerated in a cold room (4°C) to equilibrate for 24 hours. After the equilibration period, the samples were allowed to equilibrate at 25°C before the a_w of maize kernels were measured according to procedures described in Subsection 2.2.2. A curve of 'amounts of water added' against 'water activity' was constructed as illustrated in Figure 3.2.

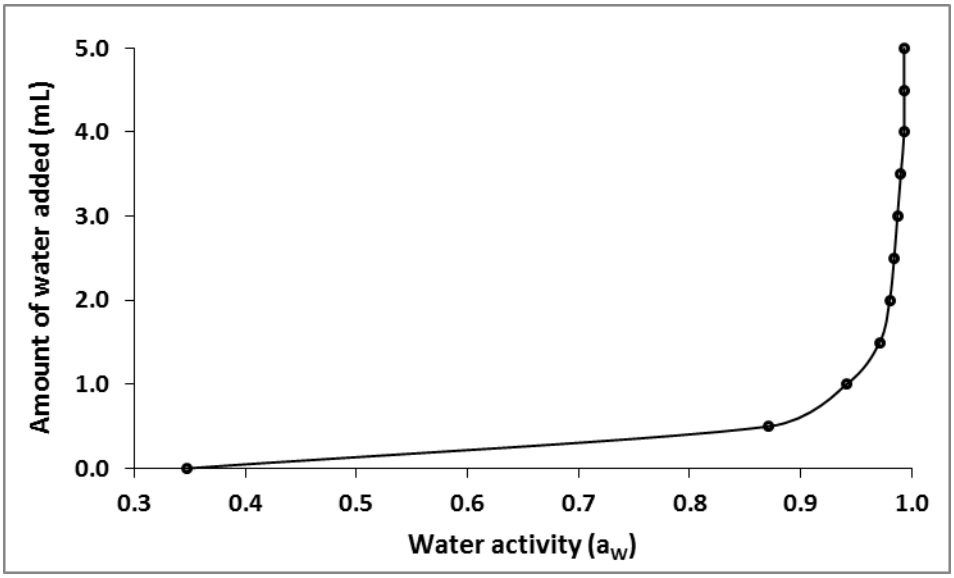

Figure 3.2. Moisture adsorption curve for 5 g maize kernels. Data are means of triplicates (n = 3). The standard errors (SE) were smaller than symbols and thus not shown on the figure.

3.2.5 Pathogen:antagonist spore ratio preparation and co-cultivation on maize agar and maize kernels

Fungal and bacterial candidates which exhibited antagonism during the preliminary screening were selected for inoculum ratio experiments, and their cultural characteristics (macro-morphology) are listed in Table 3.1. Two yeasts and one bacterium were isolated from maize kernels.

Table 3.1. List of potential biological control agents (BCAs 1-6) used to antagonize the maize pathogen *Fusarium verticillioides* FV1.

FUNGAL CANDIDATES	CODE	CULTURAL DESCRIPTION, REFERENCE
***Clonostachys rosea* 016**	**BCA1**	Dense mycelial mat with whitish hyphae. Slow grower. (Palazzini *et al.*, 2013)
Yeast II	**BCA2**	Isolated from maize kernels. Intense yellow colony with non-slimy texture.
Yeast III	**BCA3**	Isolated from maize kernels. Whitish-cream colony with non-slimy texture.
BACTERIAL CANDIDATES		
***Streptomyces* sp. AS1**	**BCA4**	Intense white colony with powdery texture (spores formation). (Sultan and Magan, 2011)
Gram-negative rod bacterium	**BCA5**	Isolated from maize kernels. Cream-coloured colony with thinly transparent texture. Expanding in size (motile).
Enterobacter hormaechei	**BCA6**	Appearing as moist and smooth cream-coloured colonies (Pereira *et al.*, 2010)

To obtain spore inoculum of FV1 and fungal candidates, 15 mL sterilized 0.1% Tween-80 solution (v/v) was transferred onto the surface of a 10-day axenic cultures of both species cultivated on PDA. A sterilized bent glass rod was used to scrape the culture surface to dislodge the fungal spores during which Tween-80 acted as a surfactant. The spore suspension was decanted into a sterile Universal bottle. For bacterial inoculum, a loopful of bacterial colony cultivated on NA was aseptically transferred using a sterilized Henle loop into 15 mL sterilized 0.1% Tween-80 solution (v/v). To estimate the number of spores in the spore suspension of both FV1 and the BCAs, a Helber haemocytometer (depth 0.02 mm; Marienfeld, Germany) was used. Steps to calculate spores from the square-grids of the haemocytometer are described in Appendix E. Spore suspension for both FV1 and BCAs were further diluted (separately) with sterilized 0.1% Tween-80 solution (v/v) to obtain $\approx 10^6$ spores/mL. A series of inoculum ratios for pathogen:antagonist were then prepared according to Table 3.2. Inoculum ratio of 100:0 (FV1 spores only) served as a negative control, while inoculum ratio of 0:100 (BCA spores only) served as a positive control.

Table 3.2. Amounts of spore suspension, and final inoculum ratios for pathogen:antagonist co-cultivation.

SPECIES	AMOUNT OF SPORE SUSPENSION (mL)					TOTAL (mL)
F. verticillioides FV1	6.0	4.5	3.0	1.5	0.0	15.0
Biocontrol candidate	0.0	1.5	3.0	4.5	6.0	15.0
Final inoculum ratio	**100 : 0**	**75 : 25**	**50 : 50**	**25 : 75**	**0 : 100**	

The co-cultivation of pathogen:antagonist on milled-maize agar (*in vitro*) at 0.95 and 0.98 a_w was done by aseptically pipetting 100 µL of each of the mixed pathogen:antagonist spore suspensions onto the surface of MMA before spreading it evenly using a sterilized bent glass rod.

The co-cultivation of pathogen:antagonist on irradiated maize kernels (*in vivo*) was done by first weighing 10 g of gamma-irradiated maize kernels before aseptically transferring the kernels into sterilized glass culture vessels equipped with microporous lid which allows air and humidity exchange (Magenta, UK). Sterilized water was then added according to Table 3.3 to modify the a_w to 0.95 and 0.98 based on the moisture adsorption curve prepared earlier. All bottles containing hydrated maize kernels were refrigerated in a cold room (4°C) with lids on to equilibrate for 24 hours. After the equilibration period, and returning treatments to ambient conditions, 1 mL of each of the five inoculum-ratio suspension prepared earlier was aseptically added to the maize kernels in the culture vessels in triplicate and mixed well.

Table 3.3. Amount of sterilized water and mixed pathogen:antagonist spore suspension for inoculation to add to the 10 g maize kernels to modify the water activities.

Water activity (a_w)	Sterilized water (mL)	Mixed-ratio suspension (mL)	Total water added (mL)
0.98	3.5	1.0	4.5
0.95	1.5	1.0	2.5

Inoculated maize agar and maize kernels were incubated separately according to a_w for 14 days at 25°C in a large sealed plastic chamber. A glycerol/water solution of the same a_w was placed in the chamber to help maintain the ERH of the atmosphere as the target a_w. Overall, both *in vitro* and *in vivo* assays consisted of 4 FV1+BCA pairs × 2 a_w × 5 inoculum ratios × 3 replicates (Σ = 120).

3.2.6 Fumonisin B_1 (FB_1) extraction from maize agar and maize kernels

For *in vitro* treatments, mycelial plugs were taken randomly from the surface of MMA plate cultures at the end of the 14-day incubation period, transferred into a 2 mL safe-lock tube (Eppendorf, Germany), and weighed on an analytical balance (Ohaus, Switzerland) with an accuracy of ± 0.01 g. The weights were recorded. Next, 1,000 µL of extraction solvent (*i.e.*, methanol:acetonitrile:water, 25:25:50 v/v) was added to the tubes and the tubes left to extract for 30 minutes. After incubation, the tubes were vortexed vigorously, and centrifuged at 10,000 rpm with a micro-centrifuge (Biofuge, Germany) for five minutes. The supernatant was carefully filtered (nylon syringe filter, 0.22 µm aperture; Jaytee, UK) into a new tube. The filtrate was evaporated to dryness at approximately 60°C, and the residue was redissolved in 500 µL of HPLC-FLD mobile phase. The flow chart for FB_1 extraction from hyphal plugs is shown in Figure 3.3.

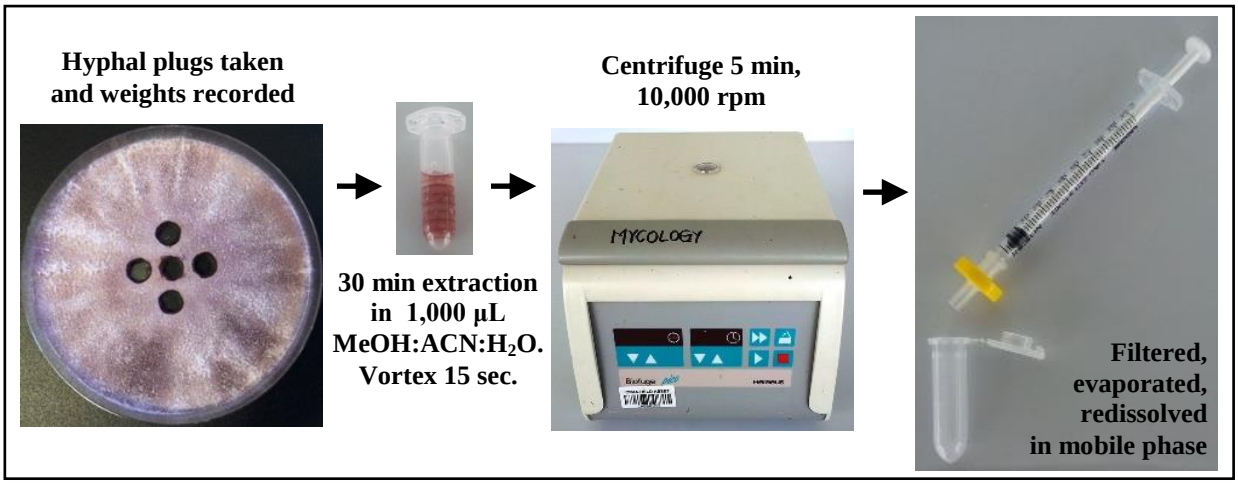

Figure 3.3. Flow diagram of FB_1 extraction from hyphal plugs on maize agar.

For *in vivo* treatments, at the end of the 14-day incubation period, fungal-colonized maize kernels were dried in the oven at approximately 75°C to remove all water content before being grinded into fine powder to increase the surface area of maize kernels. The fine powder was weighed for 10 g before adding 25 mL of extraction solvent (methanol:acetonitrile:water 25:25:50, v/v/v) and shaking them on an orbital shaker (MaxQ 5000; Thermo Scientific, USA) at 300 rpm for 30 minutes. The mixture was later centrifuged (Labofuge 400 R; Thermo Scientific, USA) for ten minutes at 3,500 *xg*, and the supernatant was filtered through Whatman® glass microfiber filters GF/A (90 mm Ø, GE Healthcare, UK) as the normal filter paper would trap a certain amount of toxin which would eventually lead to toxin underestimation. The extraction procedure was repeated with another 25 mL extraction solvent and the two extraction filtrates were pooled together. Next, 40 mL of phosphate-buffered saline (PBS) was added to 10 mL of extraction filtrate, and diluted thoroughly. PBS was prepared by dissolving 8 g NaCl, 1.8 g $Na_2HPO_4 \cdot 2H_2O$, 0.3 g KH_2PO_4, 0.2 g KCl in 1 L deionized water, and pH adjusted to 7.0 using NaOH or HCl. The diluted extract was further

filtered through a glass microfiber filter, and 10 mL of diluted filtrate (equivalent to 1/25 part of original maize powder weight) was collected for the clean-up step using the FumoniStar™ immunoaffinity columns (Item No. COIAC3000; Romer, Austria).

The immunoaffinity columns contain monoclonal antibodies against fumonisin B_1, B_2, and B_3 which will be covalently bound to gel-particles inside the column. Clean-up of 10 mL diluted filtrate (equivalent to 0.4 g maize grain) was carried out by passing it through the immunoaffinity column at a flow rate of approximately 1 mL per minute until all solution has passed through the column. After the diluted extract has completely passed through, the column was rinsed with 10 mL PBS at a flow rate of approximately 1-3 mL per minute. Immediately, without allowing time for the column to dry, elution of bound fumonisins was carried out. A suitable recipient vial was placed under the column for the collection of the eluate. The elution of bound fumonisins was carried out by passing 3 mL methanol/glacial acetic acid (98/2, v/v) through the column in several small portions (3 x 1.0 mL). The eluents were left on the column for a short period of time before letting it run off by gravity. After all eluents has passed through the column, the column was dried by passing air through with a syringe to ensure complete removal of eluents. The eluate was evaporated to dryness at approximately 60°C before the residue was redissolved in 500 µL of HPLC-FLD mobile phase. The recovery rate (%) was obtained within the range of manufacturer's specification by spiking a known concentration of FB_1 and comparing this with the results of HPLC-FLD quantification. The flow chart for FB_1 extraction and clean-up from ground powder of maize kernels is shown in Figure 3.4.

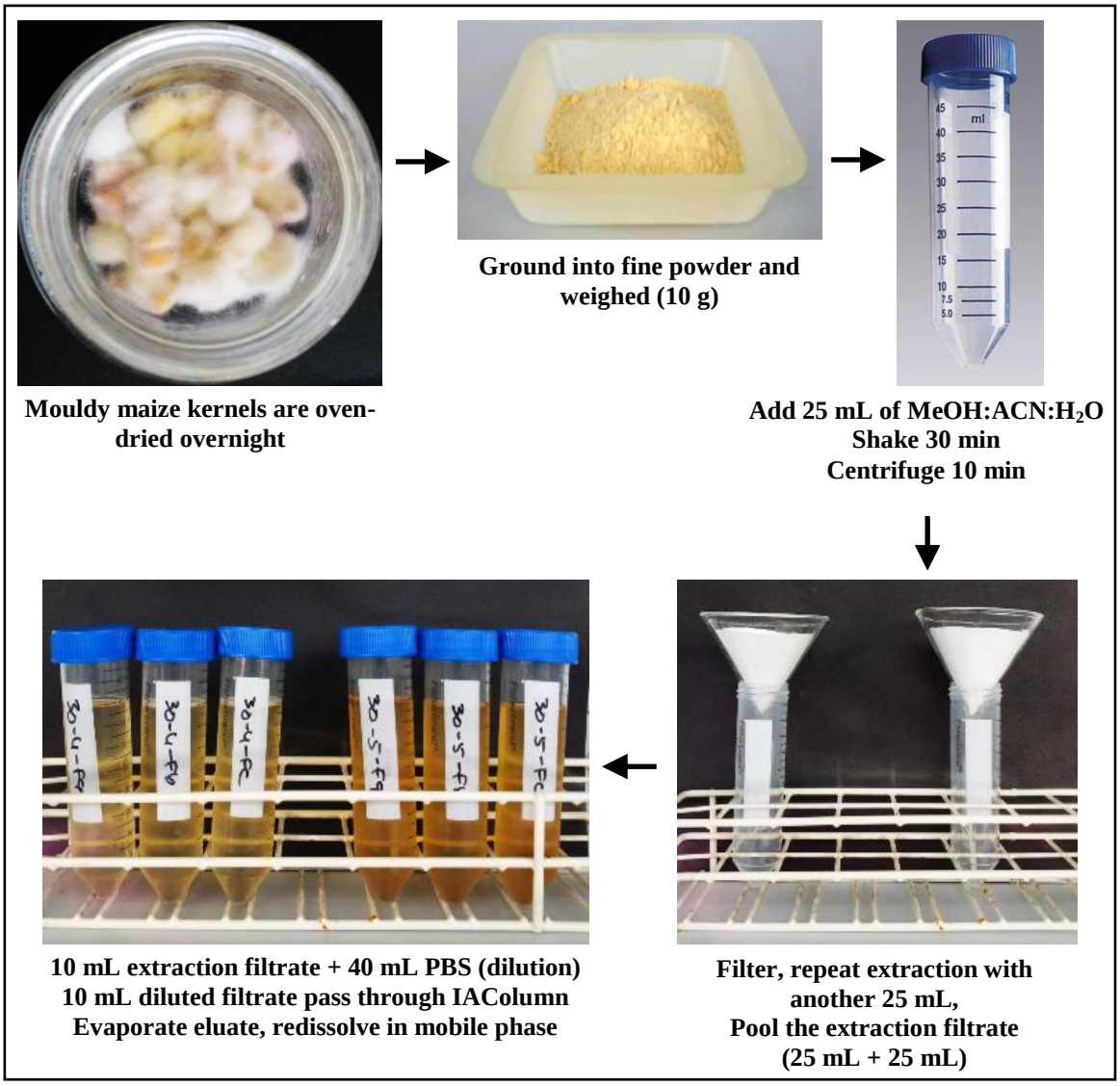

Figure 3.4. Flow diagram of FB_1 extraction and clean-up from ground powder of maize kernels.

3.2.7 Quantification of Fumonisin B_1 (FB_1) by high performance liquid chromatography joint to a fluorescence detector (HPLC-FLD)

Separation and detection of FB_1 were performed by using a reversed phase-HPLC system joined to a fluorescence detector (HPLC-FLD) with pre-column derivatization of samples with OPA. The HPLC-FLD system was as described in Subsection 2.2.6. The mobile phase was methanol:0.1 M NaH_2PO_4 (77:23, v/v), adjusted to pH 3.35 using

phosphoric acid (AOAC, 2001), filtered through a membrane (0.45µm aperture), and pumped at 1 mL per minute. The chromatographic parameters were 335 nm for the excitation wavelength (λ_{ex}) and 440 nm for the emission wavelength (λ_{em}). The injection volume was 30 µL. Before injecting the standard/sample into HPLC system, 50 µL of standard/sample was derivatized with 100 µL OPA solution within one minute. OPA solution for derivatization of standards and samples was prepared by dissolving 40 mg *ortho*-phthaldialdehyde in 1 mL absolute methanol before adding 5.0 mL 0.1 M disodium tetraborate ($Na_2B_4O_7 \cdot 10H_2O$) and 50 µL 2-mercaptoethanol in capped amber vial (light sensitive). The chemical reaction between the fumonisins and OPA is illustrated in Figure 3.5. The chromatographic data (luminescence unit peak area) were integrated and calculated using a ChemStation (Agilent, UK) software programme.

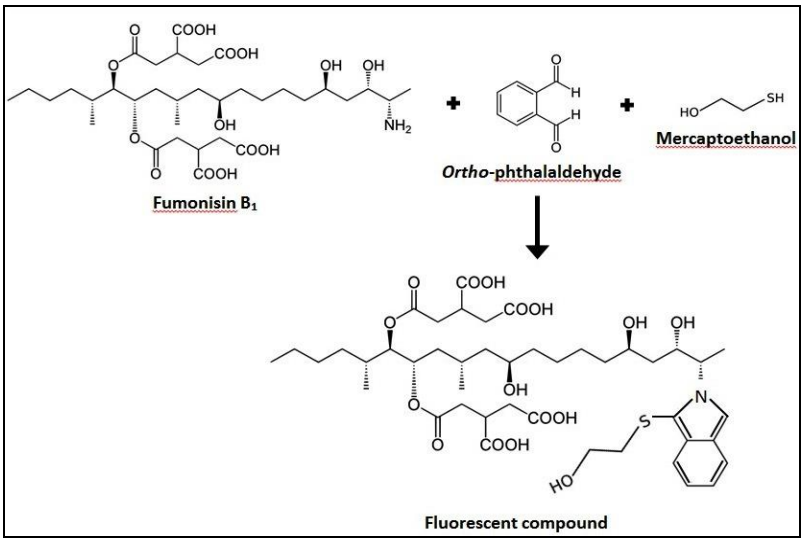

Figure 3.5. The derivatization of fumonisin B_1 with *ortho*-phthalaldehyde in the presence of mercaptoethanol which produces a fluorescent compound.

A linear standard curve was constructed to transform luminescence unit (LU) peak areas obtained through HPLC analysis into actual concentrations of fumonisin from the

hyphal plug samples (μg/g MMA) from the *in vitro* treatment, and maize powder samples (μg/g maize kernel) from the *in vivo* treatments. The analytical standard fumonisins mixture (50 μg/mL FB_1 + FB_2 in acetonitrile:water, 1:1 w/v) was purchased from Sigma-Aldrich, USA, and diluted to 0.1, 0.5, 2.5 and 5.0 μg/mL calibrant solutions. Five readings (n = 5) were recorded per each concentration of calibrant solutions (Σ = 20) intermittently throughout the samples analysis to check for reproducibility and reliability of quantification procedure. Linear standard curve was constructed by plotting the LU peak area of FBs versus concentrations of 0.1, 0.5, 2.5 and 5.0 μg/mL. The limit of detection (LOD) and limit of quantification (LOQ) were estimated by using the formulae; LOD = $3\sigma/s$ and LOQ = $10\sigma/s$, in which σ is standard deviation of y-intercepts of the FB_1 linear calibration curve, and s is the slope of the calibration curve (Medina and Magan, 2012).

3.2.8 Statistical analysis

All experiments were carried out with three replicates per treatment. Measurements were then averaged and presented as mean ± SE (standard error). Normal distribution of datasets was checked by the Kolmogorov-Smirnov normality test. Analysis of Variance (ANOVA) was applied on normally distributed datasets to analyse the variation between and within group means with 95% confidence interval. $p < 0.05$ was accepted as significantly different. Fisher's Least Significant Difference (LSD) with $\alpha = 0.05$ was applied to compare significance of differences between means of treatments using the statistical software Minitab® version 14.0 (Minitab Inc.; Pennsylvania, USA).

Figure 3.6 summarizes the key experimental steps carried out in this Chapter.

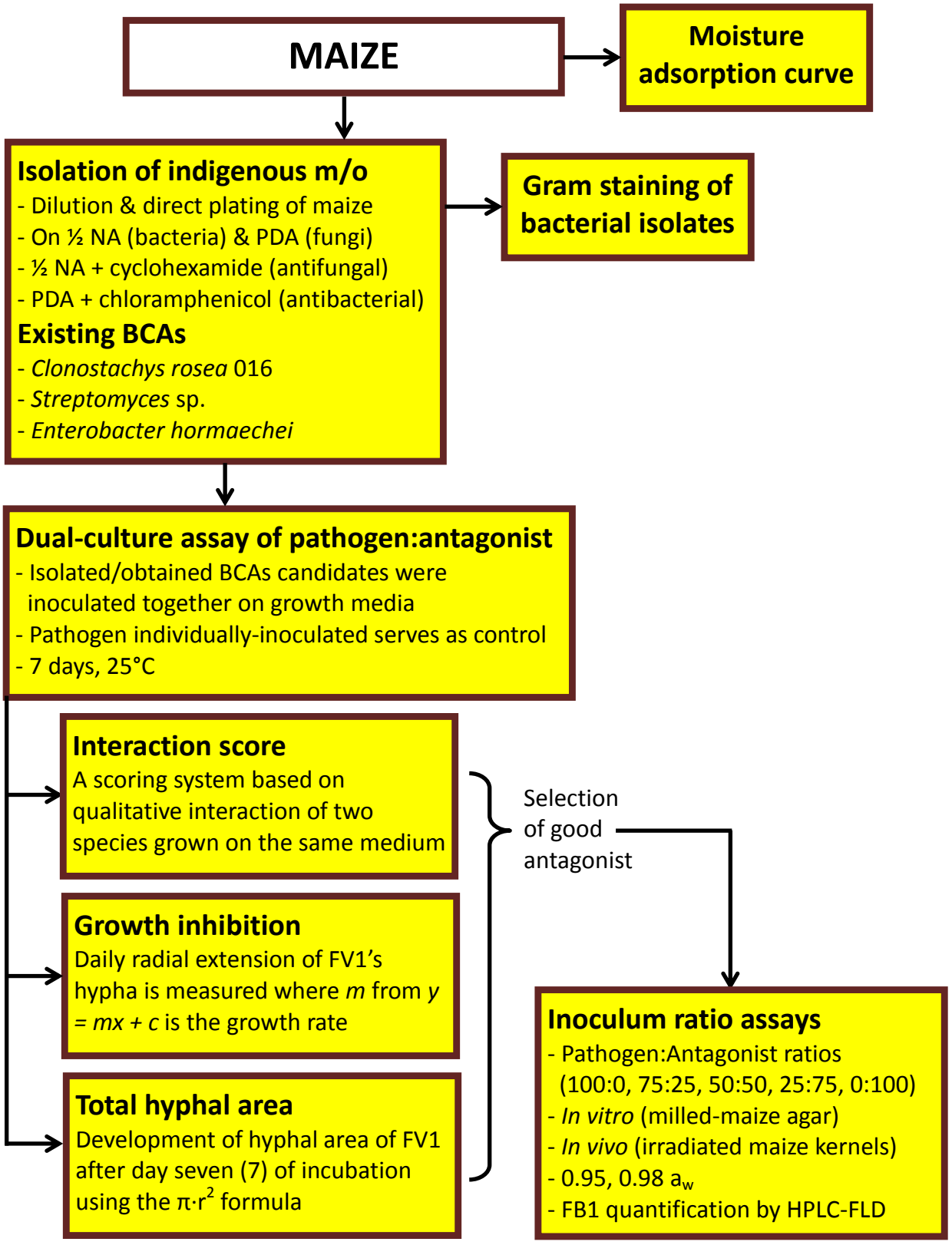

Figure 3.6. Key experimental steps carried out in Chapter 3 "Efficacy of potential biocontrol agents for control of *Fusarium verticillioides* FV1 and fumonisin B_1 under different inoculum ratios and water activities on milled-maize agar and stored maize kernels"

3.3 RESULTS

3.3.1 Screening of biological candidates in dual-culture assays

Table 3.4 shows the colony interaction scores between FV1 and the different antagonists. This shows that the fungal antagonists (BCAs 1, 2, 3) appeared to intermingle with FV1 thus showing no direct antagonism on contact. However, the bacterial antagonists were much more competitive on ½ NA where BCA4 was able to inhibit growth of FV1 at a distance, while the other two bacteria (BCAs 5, 6) antagonized FV1 on contact.

Table 3.4. Interaction scores for *Fusarium verticillioides* FV1 and antagonists (BCAs 1-6) on Potato Dextrose agar (PDA) and half-strength Nutrient agar (½ NA).

FUNGAL CANDIDATES	INTERACTION SCORES ON PDA
FV1+BCA1	1 / 1
FV1+BCA2	1 / 1
FV1+BCA3	1 / 1
BACTERIAL CANDIDATES	**INTERACTION SCORES ON ½ NA**
FV1+BCA4	0 / 5
FV1+BCA5	0 / 4
FV1+BCA6	0 / 4

Figure 3.7 shows the interactions of FV1 and BCAs 1-6 as observed in dual-culture assays and control treatments of FV1 on PDA and ½ NA. Although FV1+BCA5 plate appears as though BCA5 inhibited FV1 at a distance, the colourless zone was actually the hyaline colony of the motile BCA5 and when viewed under the microscope it showed that hyphae of FV1 was still able to grow along the periphery of the bacterial colony albeit not abundant and that no overgrowth occurred. Similar observations occurred on FV1+BCA6 plate where hyphae of FV1 were still able to grow along the periphery of the bacterial colony but without overgrowing it.

Figure 3.8 compares the growth rates of FV1 alone and in the presence of the fungal and bacterial antagonists on the two different media respectively. The growth rates of FV1 when co-cultivated with the fungal antagonists (BCAs 1, 2, 3) were not significantly different from the control ($p > 0.05$). However, the growth rates of FV1 co-cultivated with the bacterial antagonists (BCAs 4, 5, 6) showed significant difference ($p < 0.05$) from the control. Indeed, FV1+BCA5 treatment gave the lowest FV1 radial growth rates (2.75 ± 0.13 mm/d) when compared to the control, irrespective of the medium used. Although the growth of FV1 in the FV1+BCA4 and FV1+BCA6 treatments were significantly different ($p < 0.05$) from the control, they were similar to each other.

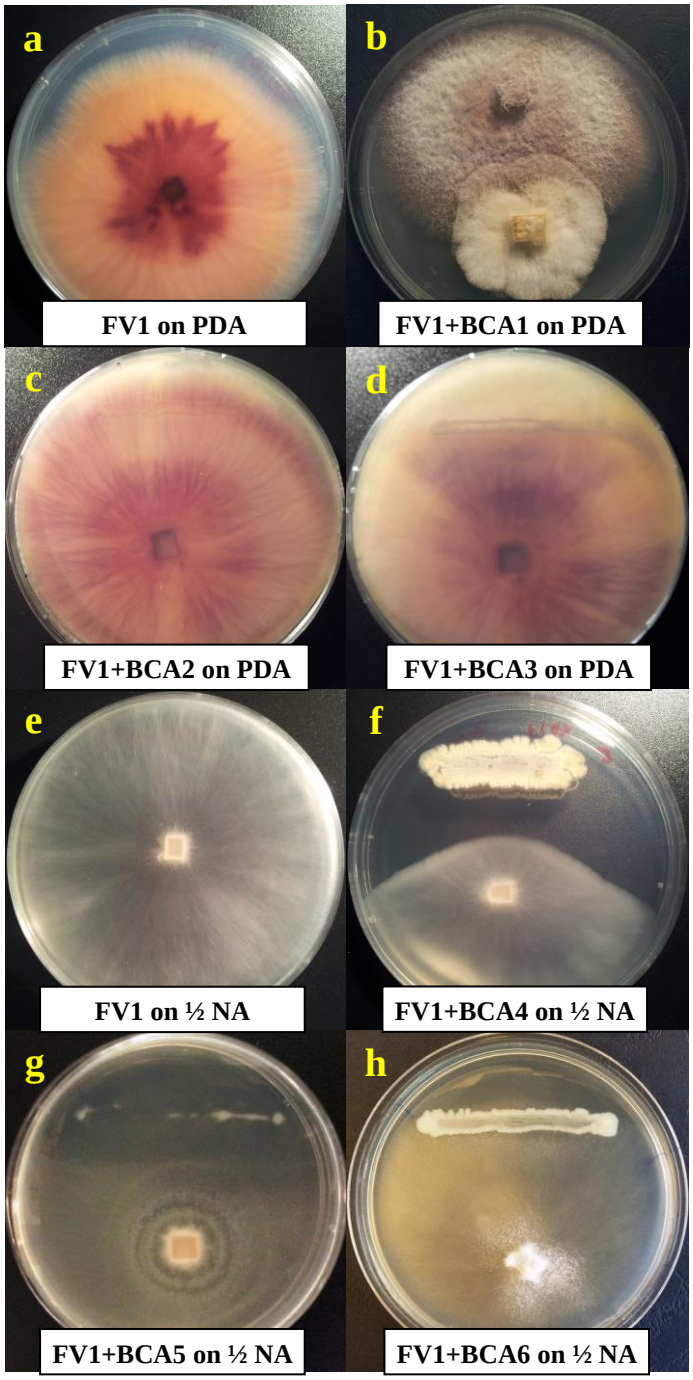

Figure 3.7. Dual-culture assays showing colony interactions of *Fusarium verticillioides* FV1 and antagonists (BCAs 1-6) on Potato Dextrose agar (PDA) and half-strength Nutrient agar (½ NA).

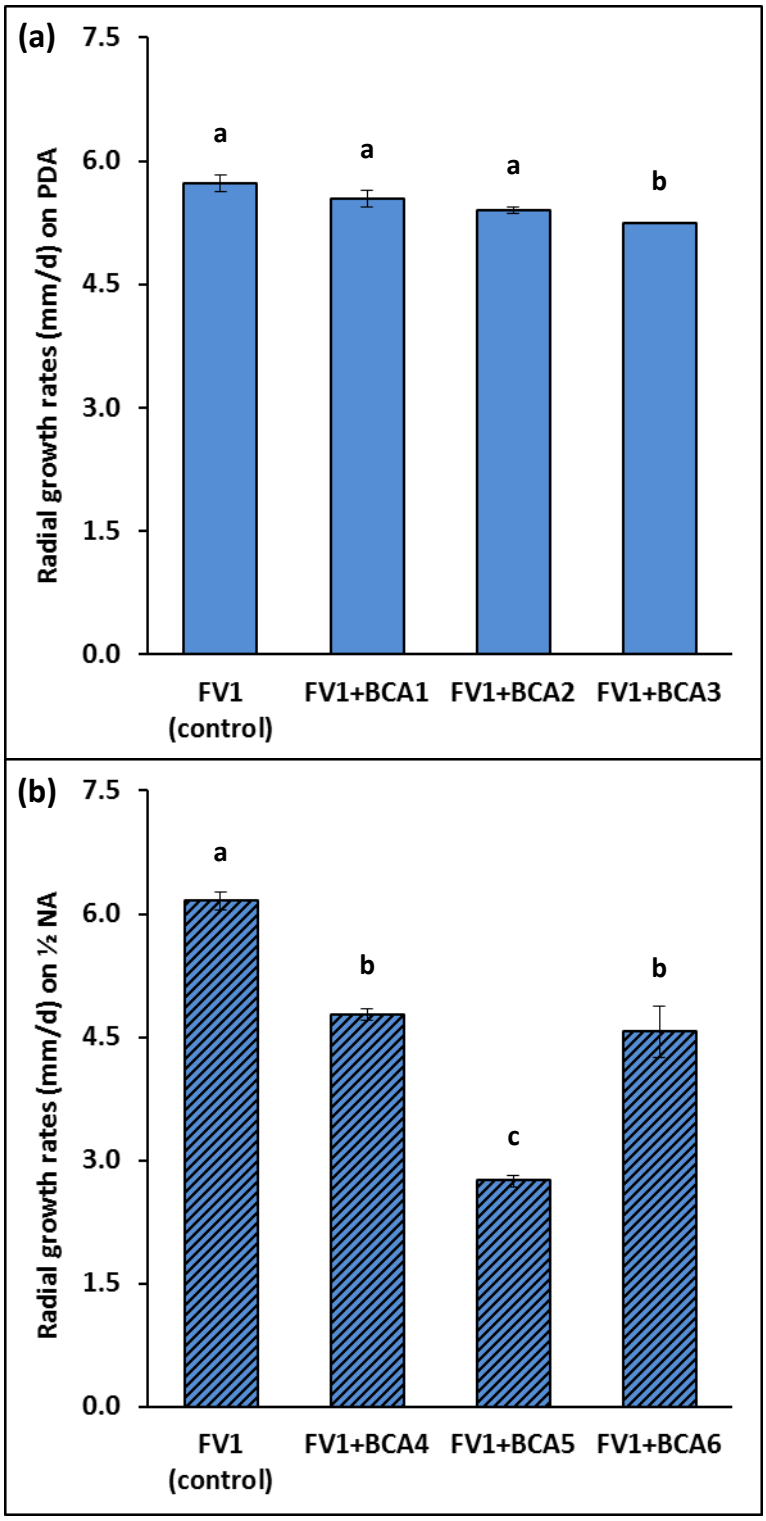

Figure 3.8. The radial growth rates (mm/d) of *Fusarium verticillioides* FV1 co-cultivated with different potential antagonists (FV1+BCAs) after seven days incubation at 25°C on (a) Potato Dextrose Agar and (b) half-strength Nutrient Agar. Data are means of triplicates (n = 3) with bars indicating standard error. Different letters indicate significant difference ($p < 0.05$) by Fisher's Least Significant Difference (LSD).

Figure 3.9 supports these results when the total hyphal area of FV1 alone and the co-cultivation with the fungal and bacterial antagonists were examined. The hyphal area of FV1 co-cultivated with the fungal antagonists (BCAs 1, 2, 3) varied with FV1+BCA2 and FV1+BCA3 being significantly different from the control. For the bacterial antagonists, all three BCAs gave significantly smaller hyphal area ($p < 0.05$) of FV1 when compared with the untreated controls. The best antagonist was BCA5 which resulted in the smallest hyphal area of FV1 (9.65 ± 1.25 cm^2). It is interesting to note that while incoherent patterns were observed in FV1 co-cultivated with fungal candidates for both growth rates and hyphal area, the pattern of hyphal area for FV co-cultivated with the bacterial candidates paralleled that of the growth rates results.

Table 3.5 list the *p*-values of the treatments as analysed by ANOVA.

Table 3.5. *P*-values for the effects of different treatments (growth media, fungal antagonists, bacterial antagonists) on the radial growth rates (mm/d) and total hyphal area (cm^2) of *Fusarium verticillioides* FV1 as analysed by Analysis of Variance (ANOVA).

	PDA	**½ NA**
Parameter	***p*-value**	
Radial growth rates of FV1	0.0084	0.0000
Total hyphal area of FV1	0.0061	0.0000

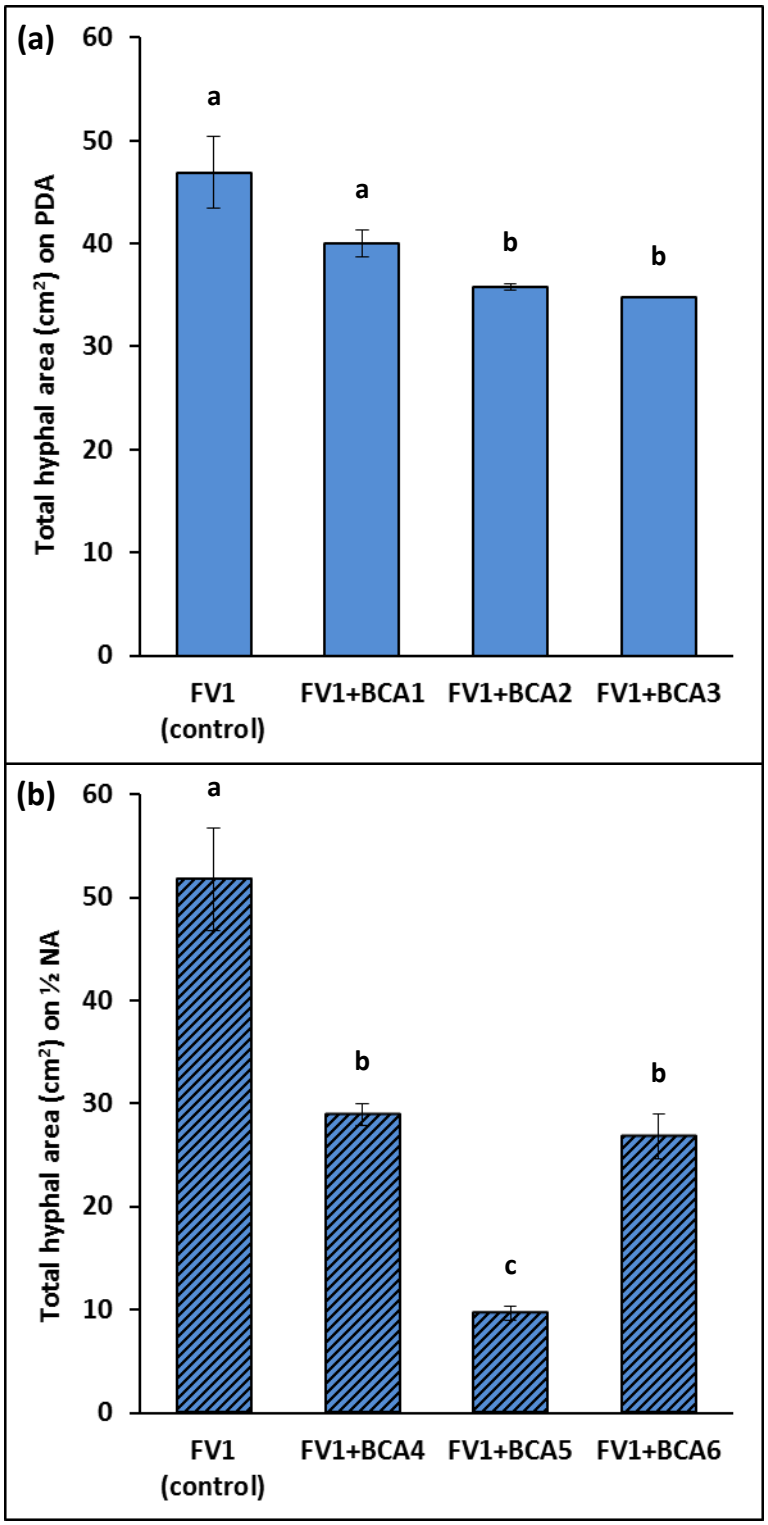

Figure 3.9. The total hyphal area (cm^2) of *Fusarium verticillioides* FV1 co-cultivated with different potential antagonists (FV1+BCAs) after seven days incubation at 25°C on (a) Potato Dextrose Agar and (b) half-strength Nutrient Agar. Data are means of triplicates ($n = 3$) with bars indicating standard error. Different letters indicate significant difference ($p < 0.05$) by Fisher's Least Significant Difference (LSD).

3.3.2 Effects of pathogen:antagonist inoculum ratios and modified water activity on FB_1 production by *Fusarium verticillioides* FV1 on maize agar

The effects of pathogen:antagonist inoculum ratios at two different a_w levels on FB_1 production *in vitro* is shown in Figure 3.10. FB_1 production was overall higher at 0.95 than at 0.98 a_w in almost all treatments, including the controls. As the ratio of pathogen to antagonist was changed, there was a significant effect on control of FB_1 achieved. Of the four antagonists examined, BCA1 (*C. rosea* 016) gave the best control resulting in complete inhibition of FB_1 production at 0.98 a_w and about 73% at 0.95 a_w. The bacterial antagonist, BCA5 (Gram-negative rod bacterium), was also very effective giving significant control irrespective of the inoculum load of the antagonist or that of the pathogen present at 78% at 0.98 a_w and 38% at 0.95 a_w on milled-maize agar. BCA4 (*Streptomyces* sp. AS1) and BCA6 (*Enterobacter hormaechei*) were also found to exhibit inhibitory properties, albeit at a lower degree. Overall, the highest amounts of FB_1 were present in the positive control treatments (FV1), and this was reduced at both a_w levels by the treatments employed. No FB_1 was found in the BCAs treatments alone (negative control). Images of the culture plates are shown in Appendix G.

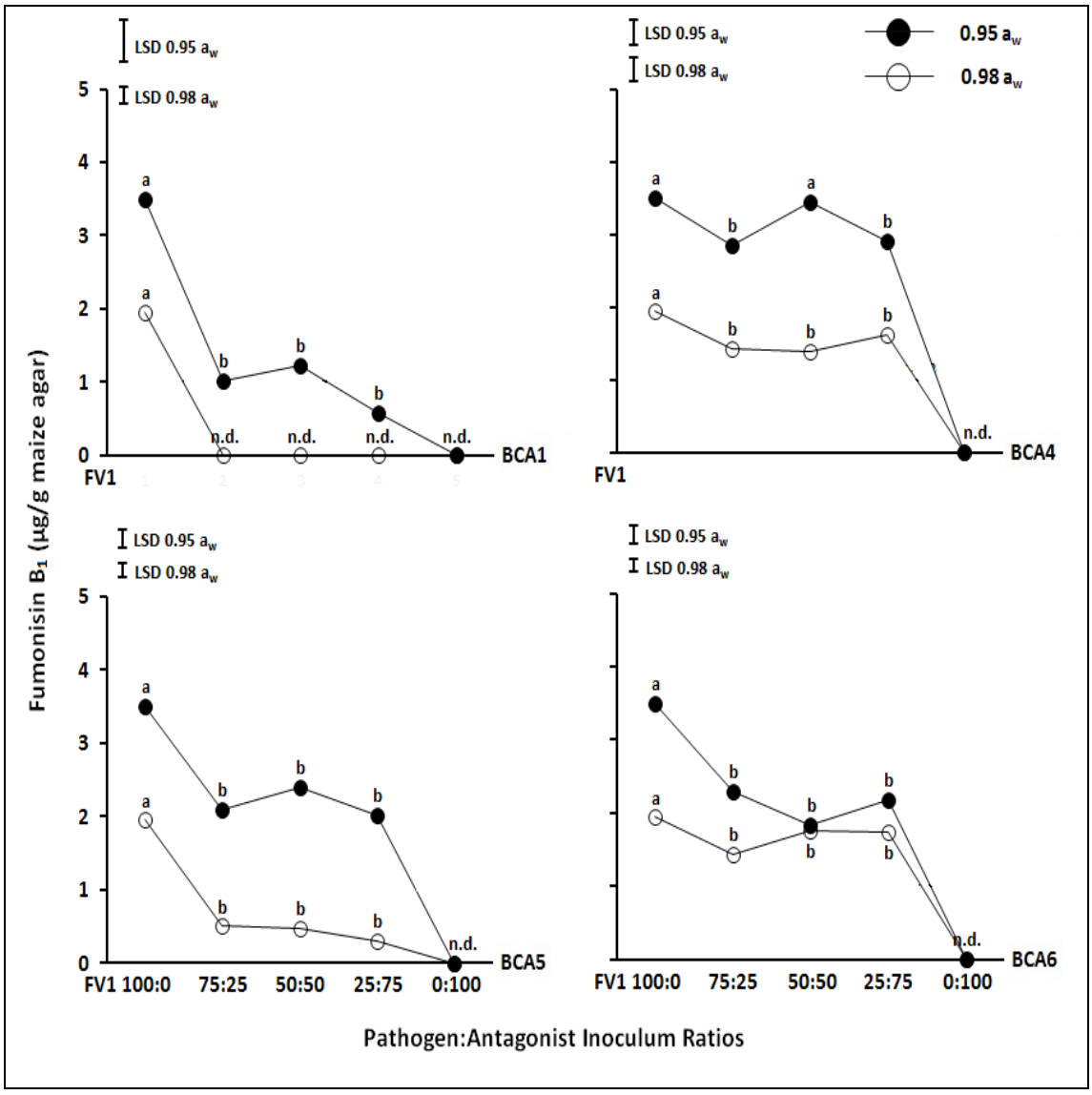

Figure 3.10. Effects of different pathogen:antagonist inoculum ratios and a_w on FB_1 production on milled-maize agar (*in vitro*). 100:0 served as positive control, and 0:100 as negative control. Dashed lines are EU limits for total FBs in maize. Data are mean of triplicate (n = 3) with bars indicating Fisher's Least Significant Difference (LSD) with $\alpha = 0.05$. Different letters indicate significant difference ($p < 0.05$) when compared to the control. n.d.; none detected. For statistical purposes, the n.d. was given zero value '0'.

Table 3.6 shows the statistical effects of a_w and inoculum ratios on FB_1 production by FV1 with BCAs *in vitro* as analysed by Analysis of Variance (ANOVA). Both a_w and inoculum ratio had significant impacts on FB_1 production as evidenced by the very low *p*-values. The interactions, although significant, were not as significant as the individual effects.

Table 3.6. *P*-values for the effects of water activities and inoculum ratios, and their interactions on FB_1 production on milled-maize agar as analysed by Analysis of Variance (ANOVA).

		FV1+ BCA1	FV1+ BCA4	FV1+ BCA5	FV1+ BCA6
Source of variation	**df[1]**	***p*-value[2]**			
Between groups[3] (water activity)	1	3.95×10^{-10}	8.69×10^{-13}	1.92×10^{-14}	2.51×10^{-7}
Within groups[4] (inoculum ratio)	4	7.62×10^{-15}	1.37×10^{-14}	9.86×10^{-15}	3.03×10^{-14}
Interaction	4	3.77×10^{-5}	1.81×10^{-6}	1.84×10^{-7}	1.74×10^{-5}

[1] Degrees of freedom. [2] Values accepted as significance at < 0.05. [3] Number of water activities (2) minus 1. [4] Number of inoculum ratios (5) minus 1.

3.3.3 Effects of pathogen:antagonist inoculum ratios and modified water activity on FB_1 production by *Fusarium verticillioides* FV1 on maize kernels

The effects of pathogen:antagonist inoculum ratios at two different a_w levels on FB_1 production *in vivo* is shown in Figure 3.11. Similar to the *in vitro* experiment, FB_1 production was in most cases significantly higher at 0.95 than at 0.98 a_w including the controls. At 0.98 a_w, BCA1 (*C. rosea* 016) gave 100% inhibition of FB_1 production at 25:75 pathogen:antagonist inoculum ratio while only 62% and 54% at 75:25 and 50:50 respectively. At 0.95 a_w, 30% stimulation in FB_1 levels were observed at 50:50 and 25:75. As for BCA4 (*Streptomyces* sp. AS1), no stimulation occurred with FB_1 inhibition observed at 63 and 55% at 0.95 and 0.98 a_w respectively. While 30% stimulation in FB_1 levels was observed at 75:25 at 0.95 a_w by BCA5 (Gram-negative rod bacterium), 70% inhibition was observed at similar ratio at 0.98 a_w. Similar to the *in vitro* treatment, BCA6 (*Enterobacter hormaechei*) *in vivo* was also found to exhibit inhibitory properties, albeit at a lower degree. Overall, the highest amounts of FB_1 were present in positive control treatments, and this was reduced at both a_w levels by some of the treatments employed. No FB_1 was found in the BCAs treatments alone (negative control). Images of the inoculated maize kernels are shown in Appendix H.

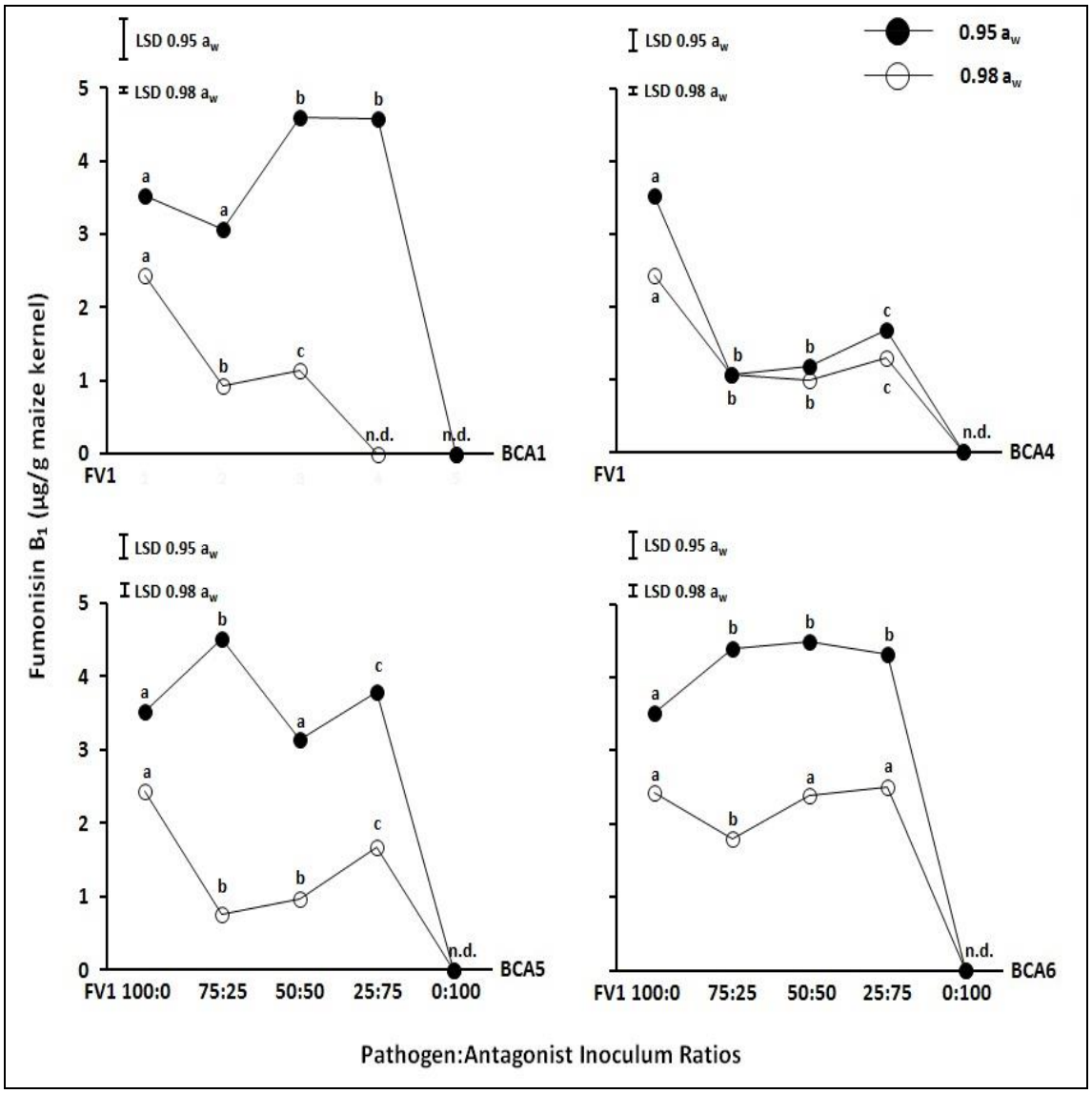

Figure 3.11. Effects of different pathogen:antagonist inoculum ratios and a_w on FB_1 production on irradiated maize kernels (*in vivo*). 100:0 served as positive control, and 0:100 as negative control. Dashed lines are EU limits for total FBs in maize. Data are mean of triplicate ($n = 3$) with bars indicating Fisher's Least Significant Difference (LSD) with $\alpha = 0.05$. Different letters indicate significant difference ($p < 0.05$) when compared to the control. n.d.; none detected. For statistical purposes, the n.d. was given zero value '0'.

Table 3.7 shows the statistical effects of a_w and inoculum ratios on FB_1 biosynthesis by FV1 with BCAs *in vivo* as analysed by Analysis of Variance (ANOVA). Similar to the effects and interactions *in vitro*, both a_w and inoculum ratio had significant impacts on FB_1 biosynthesis as evidenced by the very low *p*-values. As in *in vitro* treatments, the interactions, although significant, were not as significant as the individual factor effects.

Table 3.7. *P*-values for the effects of water activities and inoculum ratios, and their interactions on FB_1 production on irradiated maize kernels as analysed by Analysis of Variance (ANOVA).

		FV1+ BCA1	FV1+ BCA4	FV1+ BCA5	FV1+ BCA6
Source of variation	**df[1]**	***p*-value[2]**			
Between groups[3] (water activity)	1	1.22×10^{-18}	7.08×10^{-8}	6.07×10^{-19}	1.54×10^{-18}
Within groups[4] (inoculum ratio)	4	1.84×10^{-16}	2.94×10^{-20}	1.27×10^{-18}	2.15×10^{-21}
Interaction	4	3.86×10^{-14}	1.90×10^{-7}	5.85×10^{-14}	2.06×10^{-12}

[1] Degrees of freedom. [2] Values accepted as significance at < 0.05. [3] Number of water activities (2) minus 1. [4] Number of inoculum ratios (5) minus 1.

3.4 DISCUSSION

3.4.1 Effects of pathogen:antagonist inoculum ratios and modified water activity on FB_1 production by *Fusarium verticillioides* FV1 on maize agar

Of the two a_w levels used in the milled-maize agar, 0.95 a_w exhibited significantly higher FB_1 levels in all four antagonists tested as compared to 0.98 a_w which favoured growth of *F. verticillioides*. This results are in agreement with that of Jurado *et al*. (2008) and Marín *et al*. (2010) which demonstrated a decrease in growth and increase in

FBs production with increasing water stress (*i.e.*, lower a_w). This further confirms that the ecophysiological factors (*i.e.*, a_w and temperature) that allow fungal germination and growth are not necessarily the same as those that allow FBs production (Marín *et al*., 1999) which are usually over a narrower range. In this study, 0.95 and 0.98 a_w were selected for the *in vitro* treatments because these represent the a_w levels which occur in ripening maize during silking, from the milky ripe to dough stages (Zorzete *et al.*, 2008; Battilani *et al.*, 2007).

By employing the mixed cultures of different pathogen:antagonist inoculum ratios, it was possible to obtain some useful data on the threshold inoculum levels at which the antagonist was able to effectively inhibit FB_1 production by FV1. The control of FB_1 levels at both modified a_w varied in all four antagonist treatments. This was further modified by the inoculum ratios without any coherent pattern. It was found that FB_1 production was decreased at 75:25 pathogen:antagonist ratio. However, at 50:50 ratios this varied from significant inhibition to significant stimulation of FB_1 production. Nevertheless, one trend was apparent for all treatments in which the FB_1 levels of positive control (100:0) differed significantly with the next inoculum ratio (75:25) in all four antagonists tested. This clearly indicated that the introduction of antagonist spores in the inoculum however low (25% in 75:25) resulted in some inhibition of FB_1 production.

The FB_1 production levels in the present study were obtained from *in vitro* experiments. As described in the EU legislations, the stipulated limits are: 4 μg/g (≈4 ppm) FB_1+FB_2 in unprocessed maize; and 1 μg/g (≈1 ppm) FB_1+FB_2 in maize intended for direct

human consumption (EU, 2007). The *in vitro* FB_1 production in the present study was within these limits (1 – 4 μg/g). However, it should be noted that the EU limit is actually for total FBs (FB_1 + FB_2). Had the FB_2, which in nature frequently occurs together with FB_1 at levels of 15-35% of FB_1 (IARC, 2002) also been quantified, the overall FBs levels in the present study would be higher. Correspondingly, if comparable pathogenic inoculum and ecophysiological conditions were found in the field, the results might have well exceeded the EU permissible limits.

It was also noted that of all the selected BCAs tested, BCA1 managed to inhibit 100% of FB_1 production at 0.98 a_w, and significantly lowered the FB_1 levels at 0.95 a_w. As discussed earlier, higher a_w favours fungal growth. Hence, by being the only fungal antagonist, it may have an advantage in colonization of the substrate rapidly and perhaps produce inhibitory substances, or colonize the niche excluding the FV1 and thus influencing FB_1 production.

The mycoparasitic BCA1 is *Clonostachys rosea* (Sch.) Schroers and Samuels and a common world-wide soil saprophyte (Schroers, 2001; Domsch *et al*., 1980). The strain used in the present work is *C. rosea* 016. In nature, *C. rose*a is a potentially useful biocontrol agent against several economically important plant pathogens, including *Verticillium dahliae* (Keinath *et al.*, 1991), *Fusarium culmorum* (Knudsen *et al.*, 1995) and *Botrytis cinerea* (Nobre *et al.*, 2005). Some of its modes of antagonism against pathogens are nutrient competition, mycoparasitism and induced resistance (Roberti *et al.*, 2008; Sutton *et al.*, 1997). Also, its production of secondary metabolites may exert an antibiotic effect, similar to that found in other biocontrol fungi (Innocenti *et al.*,

2003; Roberti *et al*., 2002). Furthermore, other researches have described the production of fungal cell wall-degrading enzymes (*e.g.*, chitinase, glucanase; Inglis and Kawchuk, 2002; Lübeck *et al.*, 2002; Roberti *et al.*, 2002) by *C. rosea*. Several studies have used this natural advantage to develop effective biological control strategies (Butt *et al.*, 2001; Whipps, 2001). This characteristic might contribute to the inhibition of pathogenic growth in the present study and the subsequent FB_1 inhibition.

BCA5 which is a highly motile Gram-negative rod-shaped bacterium exhibited similar trends of inhibition but at a slightly lower effectiveness (78% at 0.98 a_w, 38% at 0.95 a_w). BCA4 and BCA6 were also found to exhibit inhibitory properties but at a lower degree as compared to BCA1 and BCA5.

3.4.2 Effects of pathogen:antagonist inoculum ratios and modified water activity on FB_1 production by *Fusarium verticillioides* FV1 on maize kernels

In the present work, *in vivo* experiments were performed to see whether or not the growth inhibition of FV1 and the subsequent decrease in FB_1 levels by selected BCAs achieved under *in vitro* systems occurred *in vivo*. Similar to *in vitro*, FB_1 levels *in vivo* were lower at 0.98 a_w when compared to 0.95 in the controls and in all the inoculum ratios and BCAs tested.

BCA1 exhibited lower FB_1 inhibition at both 0.95 and 0.98 a_w as compared to *in vitro* treatments. This might be attributed to the fact that BCA1 is naturally a soil-dwelling fungus. They survive as a saprophyte and colonize crop residues more effectively. *In vivo* systems which had the disadvantage of a harder substrate to colonize as compared

to *in vitro* agar surface coupled with slower growth rate of BCA1 might contribute to the lower FB_1 inhibition observed.

For the bacterial BCA4, better FB_1 inhibition was noted *in vivo* at both a_w levels tested. For BCA5 and BCA6, FB_1 inhibition were found to be similar to that *in vitro*. Bacteria are less able to grow at 0.95 a_w than fungi. This may have influenced their ability to effectively compete on stored maize kernels because of the lack of water films. This also suggests that unless damp conditions are present for enough time periods, bacteria may be more effective under very wet conditions only.

3.5 CONCLUSIONS

In summary, this study has demonstrated that the efficacy of biocontrol candidates against a mycotoxigenic species in terms of growth and colonization rates may not be a good indicator of the capacity for controlling mycotoxin production. Different pathogen:antagonist inoculum ratios were seen as having both inhibiting and stimulating effects on FB_1 production both *in vitro* and *in vivo*. The slow growing mycoparasitic BCA1 (*Clonostachys rosea* 016) completely inhibited FB_1 production at 25:75 pathogen:antagonist inoculum ratio *in vitro* and *in vivo*. BCA4 (*Streptomyces* sp. AS1) gave better inhibition *in vivo* at both a_w levels while the indigenous BCA5 (Gram-negative rod bacterium) gave better inhibition *in vitro* at both a_w levels. BCA6 (*Enterobacter hormaechei*) had comparable inhibitory effects at both a_w levels both *in vitro* and *in vivo*.

CHAPTER 4

Carbon utilization patterns and niche overlap index between potential biocontrol agents and Fusarium verticillioides FV1 under different ecophysiological conditions

4.1 INTRODUCTION

In nature, the modes of inhibition of pathogens by microbial antagonists are wide ranging (*e.g.*, competition for nutrients, synthesis of cell-wall-degrading extracellular enzymes, antibiotic/secondary metabolite production, induction of plant resistance; Benitez *et al.*, 2004). One of the most important attributes of potential biocontrol agents is the ability to compete effectively for the available nutrients in the ecological niche, and in this way colonize and exclude the pathogen. Thus, the range of nutrients which can be utilized by a potential biocontrol agent when compared to the pathogen may provide a competitive advantage and perhaps result in exclusion from a specific ecological niche. It has been suggested that the nutritional partitioning of resources in different terrestrial ecosystems (*e.g.*, phyllosphere, rhizosphere) may influence the ability of both biocontrol agents and pathogens to either co-exist or dominate in a particular niche (Wilson and Lindow, 1994; Arroyo *et al.*, 2008). In addition, the abiotic factors (*e.g.*, temperature, available water, pH) may also affect the interaction and competitiveness of both antagonist and pathogen in the occupation of an ecological niche (Magan and Aldred, 2007b). Lee and Magan (2000) showed that it was important to use a range of relevant carbon sources (C-sources) to the matrix of interest as this gives much better information on the relative C-source utilization patterns and the Niche Overlap Index (NOI) between competing microbial species. In the present study, the C-sources need to reflect those present in maize kernels.

In the laboratory, the niche overlap can be studied through the utilization pattern of C-sources which is translated in the form of Niche Overlap Index (NOI) which in turn reflects whether two strains co-exist (NOI > 0.9) or occupy separate ecological niches (NOI < 0.9). The resulting NOI would help explain the co-existence or niche exclusion by different microbial species which in turn help in the choice of appropriate biocontrol agents. However, the use of NOI alone is insufficient in understanding the nutritional patterns of competing strains. The use of the Bioscreen® to monitor the temporal rates of C-source utilization by specific biocontrol and pathogen strain has been used to try and understand the mechanism of action for mycotoxin control by antagonists (Mohale *et al.*, 2013). Such information may help in understanding the reasons for the capability of biocontrol strains to compete effectively with the pathogen, and better understand the nutritional resource partitioning under different environmental conditions in utilizing relevant C-sources.

Therefore, the objectives of this Chapter were:

(a) To identify the similarity and differences in C-source utilization patterns by the potential biocontrol agents and *F. verticillioides* FV1 under different a_w × temperature conditions

(b) To use this data to obtain Niche Overlap Indices (NOIs) between the antagonists and the pathogen under different a_w × temperature conditions

(c) To determine whether there were differences in the rate of utilization of the C-sources in maize using the Bioscreen® system to obtain the Temporal Carbon Utilization Sequence (TCUS) by the antagonists and the pathogen under different a_w × temperature conditions

4.2 MATERIALS AND METHODS

Carbon source utilization and Niche Overlap Index (NOI) studies

4.2.1 Microorganisms

A fumonisin-producing strain of *Fusarium verticillioides* FV1 which was isolated from Malaysian maize kernels and identified morphologically and molecularly as described in Subsection 2.2.5 was used in the present work. BCA1 (*Clonostachys rosea* 016) and BCA5 (Gram-negative motile bacterium) which inhibited FB_1 production *in vitro* and *in vivo* (Chapter 3) were maintained at 25°C on Malt Extract agar and Nutrient agar respectively.

4.2.2 Microtitre plate preparation

For C-source utilization patterns and determination of the Niche Overlap Indices between the antagonists and FV1, sterile 24 well microtitre plates (1 mL volume; Nunc, Denmark) were used. A minimal medium comprising (w/v) 0.23% $NaNO_3$, 0.06% $MgSO_4 \cdot 7H_2O$, 0.17% K_2HPO_4, and 0.13% KH_2PO_4 was prepared and modified to 0.95 and 0.98 a_w by adding 30 and 40% (v/v) polyethylene glycol 600 (PEG 600; Fisher, UK) respectively (Figure 4.1). The procedures to prepare the isotherm plots of PEG 600 are described in Appendix I. Glycerol was not used to modify the a_w as it is also a C-source. A total of 24 C-sources (Table 4.1) which are the principal chemical constituents of maize kernels (Giorni *et al.*, 2009) were incorporated separately into the minimal medium at a final concentration of 9.1 mg carbon mL^{-1} (carbon equivalent to 2% (w/v) glucose). The 24 C-source solutions (C-source + minimal medium) were then

autoclaved (Meadowrose, UK) at 121°C for 15 minutes at 15 psi. After cooling, the 24 C-source solutions were aseptically pipetted (700 µL) into each of the 24 wells. The type, description and molecular weight of each C-source are listed in Appendix J.

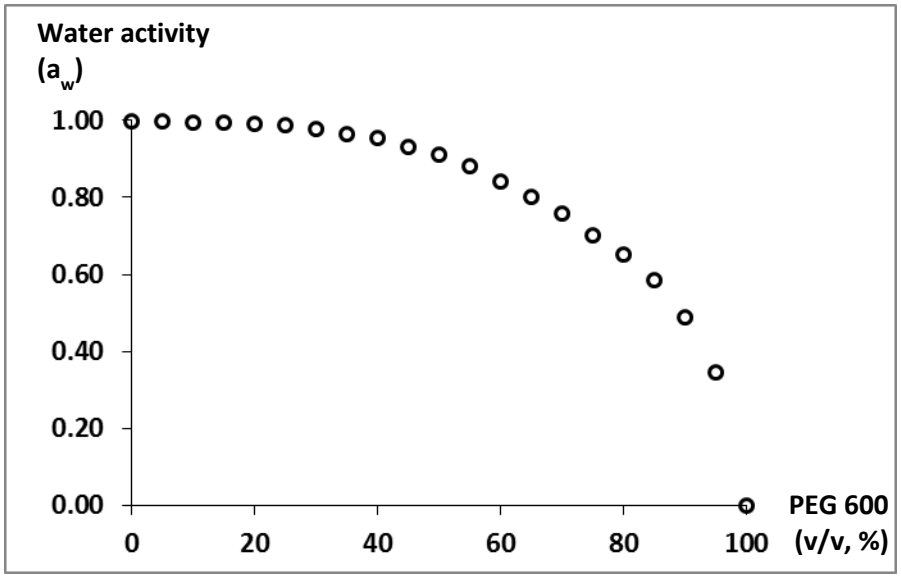

Figure 4.1. Isotherm plots for polyethylene glycol 600 (PEG 600) at 22.5°C.

4.2.3 Inoculum preparation and inoculation

Spores from seven-day old cultures grown on MEA for FV1 and BCA1, and one-day old cultures of the bacterium (BCA5) grown on NB were harvested with sterile water and aseptically transferred into separate sterile centrifuge tubes containing 20 mL of distilled water before being centrifuged for 15 minutes at 3,500 *xg*. After discarding the supernatant, they were washed three times with 20 mL sterile water before being suspended in sterile buffer solutions where the spore/cell concentration was diluted to obtain $\approx 10^6$ spores/mL by means of a Helber haemocytometer (depth 0.02 mm; Marienfeld, Germany) as described in Subsection 3.2.5 (enumeration of spore/cell). Each of the wells was inoculated with 100 µL of the spore suspension. Microtitre plates without inoculum were prepared and incubated as non-growth controls. All inoculated

plates were covered with lids and sealed with Parafilm and incubated at 25 and 30°C. The presence/absence of fungal/bacterial growth was checked with a dissecting microscope (Olympus, Japan) at 12-hour intervals for 120 hours.

Table 4.1. Carbon sources and percentage of the compound added in each well. L- and D- notations are spatial configurations of two optical isomers (enantiomers; mirror images). They are named after the Latin *dexter* (right) and *laevus* (left).

CARBON SOURCE	% COMPOUND (w/v)
Amino acids	
L-Leucine	1.65
L-Alanine	2.25
D-Alanine	2.25
D–L-Threonine	2.25
L-Serine	2.68
D-Serine	2.68
L-Histidine	1.96
L-Proline	1.74
L-Phenylalanine	2.00
L-Aspartic acid	2.00
L-Glutamic acid	2.00
Carbohydrates	
D-Galactose	2.28
D-Raffinose	2.50
D-Glucose	2.28
D-Maltose	2.28
D-Fructose	2.28
Sucrose	2.16
D-Melibiose	2.28
Dextrin	2.00
Amylopectin	2.00
Amylose	2.00
Fatty acids	
Oleic acid	2.00
Linoleic acid	2.00
Palmitic acid	2.00

4.2.4 Calculation of Niche Overlap Index (NOI)

The results of C-source utilization patterns were used to calculate the Niche Overlap Index (NOI; Wilson and Lindow, 1994). The NOI was obtained by comparing the C-source utilization patterns by the pathogen and the antagonist as shown below.

$$\mathbf{NOI} = \frac{\textit{Number of common CS used by both pathogen and antagonist}}{\textit{Number of CS used by antagonist}}$$

Equation 4.1. Formula to calculate the Niche Overlap Index (NOI) between competing strains of pathogen and antagonist (adapted from Wilson and Lindow, 1984).

The NOI values obtained are between 0 and 1, and they define whether competing strains co-exist (NOI > 0.9) or occupy separate niches (NOI < 0.9; Wilson and Lindow, 1994; Arroyo *et al.*, 2008; Mohale *et al.*, 2013).

Temporal Carbon Utilization Sequence studies (TCUS)

4.2.5 Turbidimetric assay

A Bioscreen-C® Microbiological Growth Analyzer (Labsystems, Finland) was used following the procedures described by Mohale *et al*. (2013). The spore suspension of FV1, BCA1 and BCA5 were prepared as described above. A 100-well Bioscreen® microtitre plate was aseptically loaded with 200 µL of each of the 24 C-source solutions. Each C-source solution was loaded in four different wells (Σ96 wells) before being separately inoculated with 10 µL of spore suspension of FV1, BCA1 and BCA5 (one species per plate). The remaining four wells in each plate were inoculated with

spore suspension and sterilized distilled water to act as negative controls. Experiments were conducted at 25°C and 30°C with no agitation for FV1 and BCA1, and with agitation for the bacterial BCA5. The optical density (OD) was automatically recorded at 20-minute intervals using the 600 nm filter over a seven-day period (10,080 minutes) for FV1 and BCA1, and three-day period (4,320 minutes) for BCA5. Data were recorded using the software Easy Bioscreen Experiment (EZExperiment).

4.2.6 Calculation of Temporal Carbon Utilization Sequence (TCUS)

Before analyses, the average of the measurements for each well during the first 60 minutes was calculated and automatically subtracted from all subsequent measurements in order to remove the different signal backgrounds obtained for the different C-source solutions. The "Time To Detection" (TTD) for an OD of 0.1 were measured at 25°C and 30°C at 0.95 and 0.98 a_w by using a linear interpolation between successive OD readings to predict the TTD at OD = 0.1. TTD for each replicate of each C-source was recorded. The average of these values, expressing the mean time that the strains needed to reach an OD of 0.1 with a particular C-source was computed. Data for pathogen and antagonists were then organized according to these TTD values, and thus the TCUS were obtained (Mohale *et al.*, 2013).

4.2.7 Statistical analysis

All experiments were carried out with three replicates for C-source utilization patterns and for determining the NOI; and four replicates for TCUS per treatment (a_w × temperature) per species. All experiments were repeated once. Measurements were then averaged and presented as mean ± SE (standard error). Normal distribution of datasets

was checked by the Kolmogorov-Smirnov normality test. Analysis of Variance (ANOVA) was applied on normally distributed datasets to analyse the variation between and within group means with 95% confidence interval. $p < 0.05$ was accepted as significant difference. Fisher's Least Significant Difference (LSD) with $\alpha = 0.05$ was applied to compare significant difference between means of treatments; available water, temperature, and carbon source using the statistical software Minitab® version 14.0 (Minitab Inc.; Pennsylvania, USA).

Figure 4.2 summarizes the key experimental steps carried out in this Chapter.

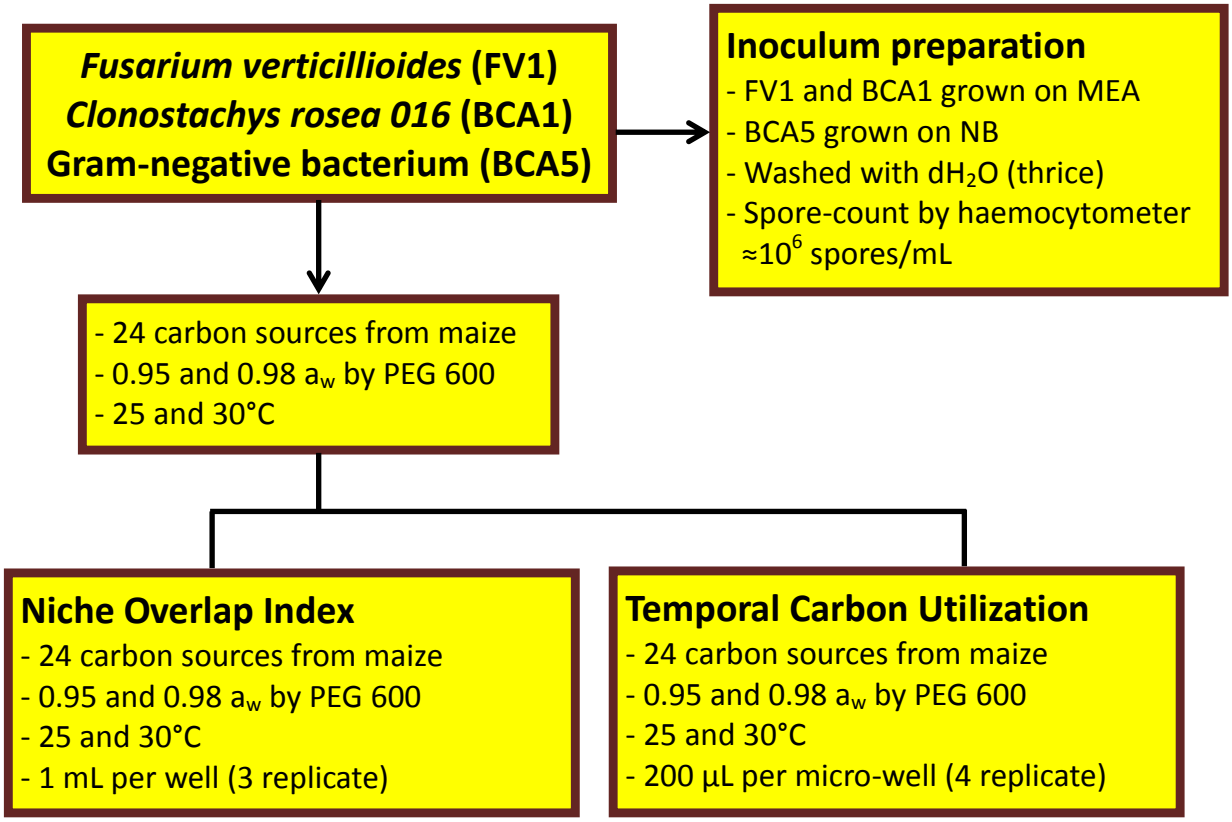

Figure 4.2. Key experimental steps carried out in Chapter 4 "Carbon utilization patterns and niche overlap index between potential biocontrol agents and *Fusarium verticillioides* FV1 under different ecophysiological conditions".

4.3 RESULTS

4.3.1 Carbon source utilization patterns and Niche Overlap Indices between antagonists and *Fusarium verticillioides* FV1

At the end of the incubation period, the Gram-negative BCA5 did not appear to have grown at all based on examination using a binocular low power microscope. Despite repeating the assay a number of times, BCA5 did not appear to be able to utilize the C-sources in maize kernels, irrespective of temperature or a_w used. This may be caused by the toxicity effects in the use of PEG 600 although this was not confirmed. Therefore, the following results and discussion were focussed on *Clonostachys rosea* 016 (BCA1) and *F. verticillioides* FV1.

Figure 4.3 shows the total number of C-sources (CSs) utilized by FV1 and BCA1 at different temperatures × a_w levels after seven days incubation. For FV1 at 25°C, an equal number (19) of CSs were utilized irrespective of the a_w level while at 30°C, the highest number of CSs (21) was utilized at 0.95 a_w while at 0.98 a_w the lowest number were utilized (18). For BCA1, an equal number (10) of CSs were utilized at 30°C irrespective of the a_w level. A lower number of CSs were utilized at 25°C at 0.95 a_w (8) and 0.98 a_w (5). Overall, BCA1 utilized almost half the number of CSs when compared to FV1.

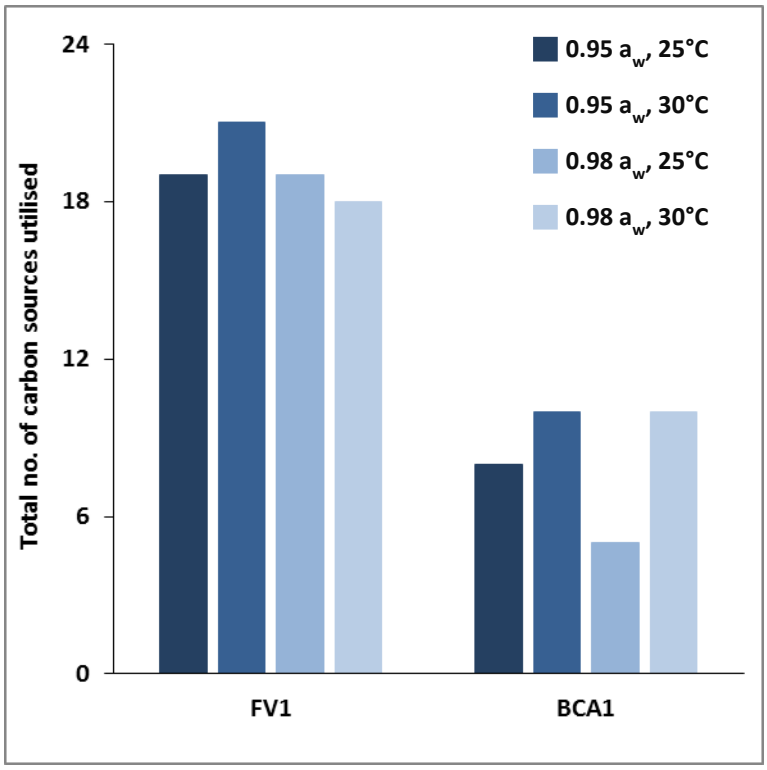

Figure 4.3. Effects of water activity × temperature on the total number of carbon sources utilized by the pathogen *Fusarium verticillioides* FV1 and the antagonist *Clonostachys rosea* 016 (BCA1).

Table 4.2 shows the *p*-values on the effects of treatments and species on the number of CSs utilization. In FV1, insignificant difference (p = 0.0506) was obtained between all treatments. In BCA1, a significant difference (p = 0.0000) was obtained between treatments with 0.98 a_w+25°C giving the lowest number of CSs. Between FV1 and BCA1, significant difference (interaction p = 0.0002) was obtained with BCA1 utilizing significantly lower CSs at all treatments when compared to FV1.

Table 4.2. *P*-values for the effects of species and treatments on the number of carbon sources utilization as analysed by Analysis of Variance (ANOVA).

FV1

Source of variation	SS[1]	df[2]	MS[3]	F	*p*-value	F crit
Between treatments	8.25	3	2.75	3.4737	0.0506	3.4903
Within treatments	9.5	12	0.7917			
Total	17.75	15				

BCA1

Source of variation	SS	df	MS	F	*p*-value	F crit
Between treatments	62.75	3	20.92	50.2	0.0000	3.4903
Within treatments	5	12	0.4167			
Total	67.75	15				

FV1 × BCA1

Source of variation	SS	df	MS	F	*p*-value	F crit
Species	1152	1	1152	1906.76	0.0000	4.2597
Treatments	52.75	3	17.5833	29.1035	0.0000	3.0088
Interaction	18.25	3	6.0833	10.069	0.0002	3.0088
Within	14.5	24	0.6042			
Total	1237.5	31				

[1] Sum of Squares, [2] Degrees of Freedom, [3] Mean of Squares

Table 4.3 shows the relative NOI values for the antagonist and the pathogen at different a_w × temperature conditions. This shows that BCA1 and FV1 occupied similar niches (co-existed) at 25°C+0.98 a_w and 30°C+0.95 a_w, and occupied different niches in other treatments.

Table 4.3. Niche Overlap Index (NOI) between the antagonist *Clonostachys rosea* 016 (BCA1) and the mycotoxigenic pathogen *Fusarium verticillioides* FV1 grown at different water activities and temperatures.

Temperature \ Water activity	0.95 a_w	0.98 a_w
25°C	0.88	0.90
30°C	0.90	0.80

Table 4.4 further compares the percentage of different types of CSs utilized by both BCA1 and FV1. Overall, FV1 utilized a higher percentage of the amino acids than carbohydrates while the antagonist utilized more carbohydrates and less amino acids under the ecophysiological conditions tested. Fatty acids were the least preferred CSs by both antagonist and pathogen.

Table 4.4. Mean percentage of carbon sources utilization according to types by the mycotoxigenic pathogen *Fusarium verticillioides* FV1 and the antagonist *Clonostachys rosea* 016 (BCA1) grown at different water activities and temperatures.

	FV1			BCA1		
	AA[1]	CH[2]	FA[3]	AA	CH	FA
0.95 a_w, 25°C	100	80	0	9.1	70	0
0.95 a_w, 30°C	100	90	33.3	27.3	70	0
0.98 a_w, 25°C	100	80	0	18.2	30	0
0.98 a_w, 30°C	90.9	80	0	45.5	50	0

[1]AA = amino acid (11 sources), [2]CH = carbohydrate (10 sources), [3]FA = fatty acid (3 sources)

4.3.2 Temporal Carbon Utilization Sequence Studies

In the TCUS studies, the Gram-negative BCA5 exhibited very low detection of cellular growth (0.001-0.090 OD by the end of three-day incubation). It was therefore assumed that PEG 600 might have toxicity effect on the bacterial growth as might have occurred in NOI studies. Therefore, the Figures 4.4 and 4.5 only illustrate the TCUS of the antagonist (BCA1) and the pathogen (FV1) respectively in different a_w × temperature conditions. In general, the time to detection (TTD) of 0.1 optical density (OD) for FV1 was shorter than that of the antagonist. It is also noteworthy that all the carbohydrate sources were utilized by FV1 by 5,500 min in all the other treatments with the single exception of amylose at 0.95 a_w and 30°C, while amino acid sources were utilized up to 9,000 min. The only fatty acid utilized was palmitic acid at 0.95 a_w and 30°C which was utilized by day 9,800 min. On average, 25°C+0.98 a_w gave 5,500 ± 500 TTD; max 9,500. When water stress was introduced at 0.95 a_w, the average TTD was reduced to 5,000 ± 425; max 9,100. At 30°C however, the opposite occurred in which 0.95 a_w gave the longer TTD at 4,800 ± 433; max 9,800 when compared to 0.98 a_w at 4,000 ± 300; max 6,500. Furthermore, across the temperatures, 30°C gave shorter TTD as compared to 25°C at both a_w levels tested. It was also apparent that the acidic amino acid (glutamic acid, aspartic acid) utilization was affected by the treatment regimes. At 0.98 a_w at both temperatures, glutamic acid was the first amino acid to be utilized while aspartic acid gave longer TTD. However, when water stress was imposed (0.95 a_w), aspartic acid was utilized earlier at both temperatures while glutamic acid took almost double (30°C) to triple (25°C) the time to reach similar OD (0.1). Similar occurrence was also observed in the utilization of starch sources. At 0.98 a_w for both temperatures, amylose was the first carbohydrate to be utilized while amylopectin remained

unutilized. However, when water stress was imposed (0.95 a_w), amylopectin was the first carbohydrate to be utilized while amylose remained unutilized (25°C) or took three times the time (30°C) to reach a similar OD (0.1). For amino acids where both enantiomers used (D- and L-alanine, D- and L-serine), it was observed that the D-isomers were utilized faster (shorter TTD) than the L-isomers under all treatment regimes.

In the case of BCA1, although more carbohydrates were utilized more rapidly when compared to amino acids, the TTD for both types of sources were up to 9,500 min. No fatty acid was utilized by BCA1 during the incubation period. Furthermore, while dextrin was not utilized at all by FV1, BCA1 was able to assimilate this type of starch. The preference over amylose-amylopectine too was shown to be the opposite of that for FV1 where BCA1 preferred amylose at lower a_w levels (0.95 a_w). The disaccharide sucrose and D-melibiose were utilized in all treatment regimes. In addition, at 0.95 a_w the monosaccharide D-glucose and the disaccharide D-maltose were also utilized.

To better visualize the increase/decrease of TTD for each CS across the treatment regimes, the TTD in ascending order (shorter to longer time) for FV1 and BCA1 are tabulated in Tables 4.5 and 4.6 respectively.

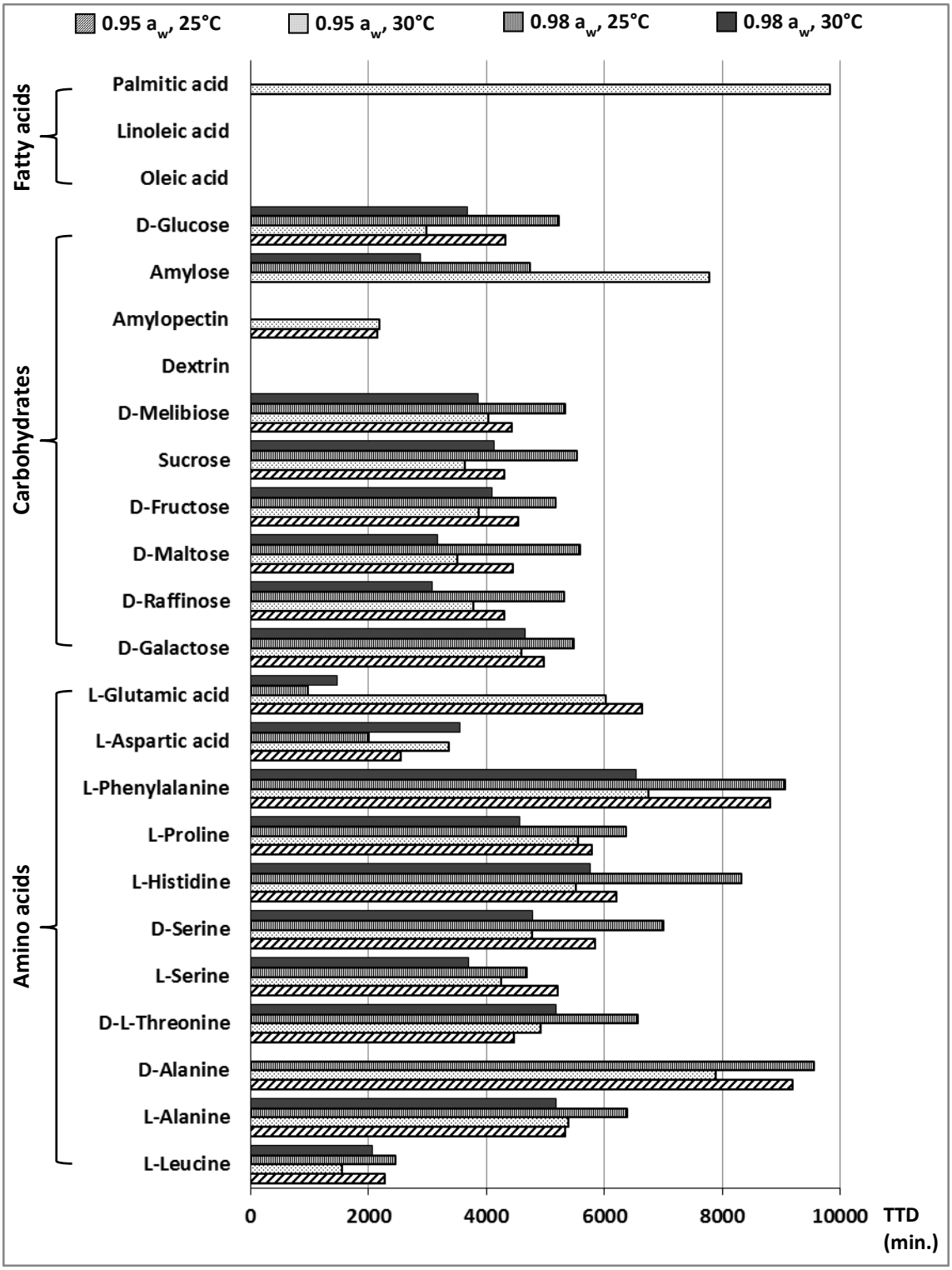

Figure 4.4. Effect of water activity (0.95, 0.98 a_w) and temperature (25°C, 30°C) on the time to detection (TTD) of the pathogen *Fusarium verticillioides* FV1 grown from spores on 24 carbon sources over seven days.
FA = fatty acids, CH = carbohydrates, AA = amino acids

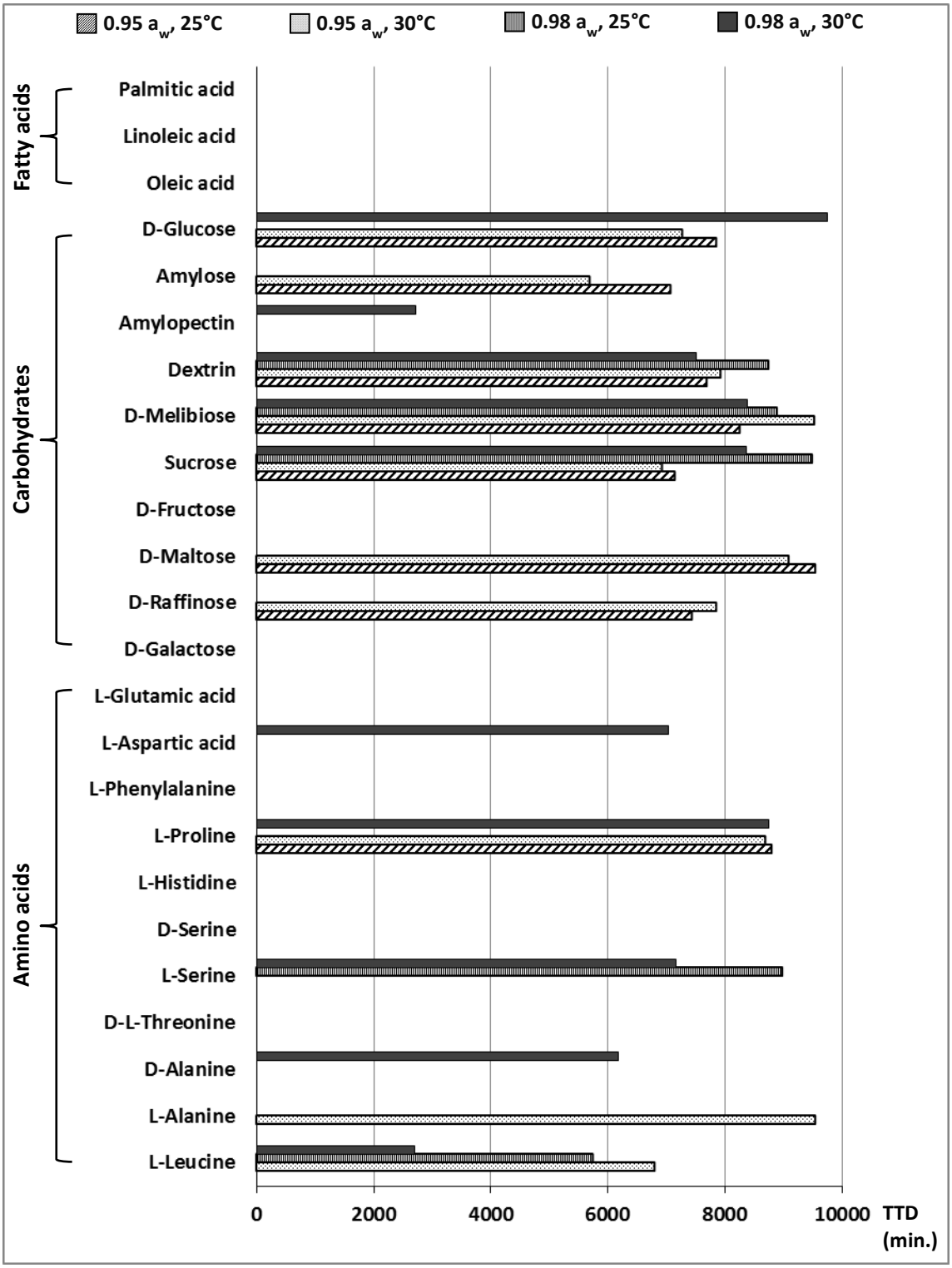

Figure 4.5. Effect of water activity (0.95, 0.98 a_w) and temperature (25°C, 30°C) on the time to detection (TTD) of the antagonist *Clonostachys rosea* 016 (BCA1) grown from spores on 24 carbon sources for seven days.
FA = fatty acids, CH = carbohydrates, AA = amino acids

Table 4.5. Visualization of time to detection (TTD) in ascending order for the 24 carbon sources (CSs) under different water activity × temperature conditions tested for growth of *Fusarium verticillioides* FV1 after seven days incubation.
Colour code: yellow = amino acids, blue = carbohydrates, red = fatty acids.
Zero TTD (0) signifies TTD above the timeframe (> 10,800 min).

0.98 a_w, 25°C		0.95 a_w, 25°C		0.98 a_w, 30°C		0.95 a_w, 30°C	
L-Glutamic acid	970	Amylopectin	2145	L-Glutamic acid	1465	L-Leucine	1555
L-Aspartic acid	2015	L-Leucine	2275	L-Leucine	2060	Amylopectin	2185
L-Leucine	2455	L-Aspartic acid	2550	Amylose	2885	D-Glucose	2990
L-Serine	4680	D-Raffinose	4305	D-Raffinose	3080	L-Aspartic acid	3375
Amylose	4747	Sucrose	4310	D-Maltose	3165	D-Maltose	3505
D-Fructose	5180	D-Glucose	4320	L-Aspartic acid	3555	Sucrose	3640
D-Glucose	5230	D-Melibiose	4430	D-Glucose	3675	D-Raffinose	3780
D-Raffinose	5325	D-Maltose	4455	L-Serine	3685	D-Fructose	3865
D-Melibiose	5345	D-L-Threonine	4475	D-Melibiose	3855	D-Melibiose	4035
D-Galactose	5480	D-Fructose	4535	D-Fructose	4090	L-Serine	4255
Sucrose	5535	D-Galactose	4975	Sucrose	4120	D-Galactose	4590
D-Maltose	5595	L-Serine	5215	L-Proline	4555	D-Serine	4770
L-Proline	6370	L-Alanine	5345	D-Galactose	4650	D-L-Threonine	4925
L-Alanine	6395	L-Proline	5800	D-Serine	4775	L-Alanine	5385
D-L-Threonine	6567	D-Serine	5850	D-L-Threonine	5175	L-Histidine	5520
D-Serine	7000	L-Histidine	6200	L-Alanine	5180	L-Proline	5550
L-Histidine	8330	L-Glutamic acid	6640	L-Histidine	5755	L-Glutamic acid	6025
L-Phenylalanine	9073	L-Phenylalanine	8805	L-Phenylalanine	6535	L-Phenylalanine	6745
D-Alanine	9550	D-Alanine	9193			Amylose	7780
				D-Alanine	0	D-Alanine	7893
Dextrin	0	Dextrin	0	Dextrin	0	Palmitic acid	9820
Amylopectin	0	Amylose	0	Amylopectin	0		
Oleic acid	0	Oleic acid	0	Oleic acid	0	Dextrin	0
Linoleic acid	0	Linoleic acid	0	Linoleic acid	0	Oleic acid	0
Palmitic acid	0	Palmitic acid	0	Palmitic acid	0	Linoleic acid	0

Table 4.6. Visualization of time to detection (TTD) in ascending order for the 24 carbon sources (CSs) under different water activity × temperature conditions tested for growth of *Clonostachys rosea* 016 (BCA1) after seven days incubation.
Colour code: yellow = amino acids, blue = carbohydrates, red = fatty acids.
Zero TTD (0) signifies TTD above the timeframe (> 10,800 min).

0.98 a_w, 25°C		0.95 a_w, 25°C		0.98 a_w, 30°C		0.95 a_w, 30°C	
L-Leucine	5747	Amylose	7080	L-Leucine	2695	Amylose	5707
Dextrin	8760	Sucrose	7160	Amylopectin	2720	L-Leucine	6807
D-Melibiose	8910	D-Raffinose	7445	D-Alanine	6170	Sucrose	6930
L-Serine	9000	Dextrin	7693	L-Aspartic acid	7040	D-Glucose	7290
Sucrose	9495	D-Glucose	7860	L-Serine	7155	D-Raffinose	7865
		D-Melibiose	8260	Dextrin	7500	Dextrin	7935
L-Alanine	0	L-Proline	8820	Sucrose	8360	L-Proline	8710
D-Alanine	0	D-Maltose	9560	D-Melibiose	8380	D-Maltose	9095
D-L-Threonine	0			L-Proline	8747	D-Melibiose	9535
D-Serine	0	L-Leucine	0	D-Glucose	9740	L-Alanine	9553
L-Histidine	0	L-Alanine	0				
L-Proline	0	D-Alanine	0	L-Alanine	0	D-Alanine	0
L-Phenylalanine	0	D-L-Threonine	0	D-L-Threonine	0	D-L-Threonine	0
L-Aspartic acid	0	L-Serine	0	D-Serine	0	L-Serine	0
L-Glutamic acid	0	D-Serine	0	L-Histidine	0	D-Serine	0
D-Galactose	0	L-Histidine	0	L-Phenylalanine	0	L-Histidine	0
D-Raffinose	0	L-Phenylalanine	0	L-Glutamic acid	0	L-Phenylalanine	0
D-Maltose	0	L-Aspartic acid	0	D-Galactose	0	L-Aspartic acid	0
D-Fructose	0	L-Glutamic acid	0	D-Raffinose	0	L-Glutamic acid	0
Amylopectin	0	D-Galactose	0	D-Maltose	0	D-Galactose	0
Amylose	0	D-Fructose	0	D-Fructose	0	D-Fructose	0
D-Glucose	0	Amylopectin	0	Amylose	0	Amylopectin	0
Oleic acid	0	Oleic acid	0	Oleic acid	0	Oleic acid	0
Linoleic acid	0	Linoleic acid	0	Linoleic acid	0	Linoleic acid	0
Palmitic acid	0	Palmitic acid	0	Palmitic acid	0	Palmitic acid	0

4.4 DISCUSSION

Comparisons of the relative utilization of C-sources by the fungal biocontrol agent and the pathogen showed distinct differences in the total number and the types used under the different a_w × temperature conditions examined. These differences might be explained by the fact that BCA1 is known to colonize matrices relatively slowly (≈40 mm diameter after seven days) when compared with the growth of FV1 (≈80 mm diameter after seven days).

It is interesting to note that while FV1 predominantly utilized higher numbers of amino acids, BCA1 utilized higher numbers of carbohydrates. The subsequent NOI values generated, based on the number of common CSs utilized by both species, showed that they occupied the same nutritional niche at 0.95 a_w+30°C and 0.98 a_w+25°C. When the treatment regimes were changed, the NOI values were modified, suggesting occupation of separate niches. The present findings are different from those of Giorni *et al*. (2009) who investigated the C-source utilization patterns of maize nutrients for a strain of *F. verticillioides* (ITEM 1744) at 25 and 30°C. They found that the fumonisin producing strain utilized a significantly higher number of CSs at 25°C than at 30°C. However, this difference might be attributed to strain differences. The study by Giorni *et al*. (2009) was not aimed at comparing potential biocontrol agents but the relative occupation of the maize niche by different mycotoxigenic fungi under different ecophysiological conditions. The present study has thus provided data on the ability of the antagonist BCA1 to utilize different C-sources relevant to maize for the first time.

In the natural maize agro-ecosystem, temperatures of 20-30°C are typical between flowering and harvest, and a_w values of 0.87 to 0.98 a_w can occur in maize cobs during early dough to full ripe stage (Zorzete *et al.*, 2008; Battilani *et al.*, 2007). Both FV1 and BCA1 can colonize maize substrates at > 0.93 a_w. Thus there is an opportunity for establishment of the biocontrol agent to compete with the pathogen during silking.

Previously, the use of *C. rosea* strains as a mycoparasite of several strains of economically important fungal phytopathogens has been described. For example, it has been shown to control the growth of *F. culmorum* and *F. graminearum* on wheat, *F. culmorum, F. graminearum* and *F. proliferatum* on maize (Palazzini *et al.*, 2013; Luongo *et al.*, 2005), *Sclerotinia sclerotiorum* on soybean and lettuce (Rodríguez *et* al., 2011), *Moniliophtora roreri* and *Phytophtora* spp. on cocoa (Mejía *et al*., 2008), *Alternaria* spp. on carrot (Jensen *et al.*, 2004), *Bipolaris sorokiniana* on barley (Jensen *et al.*, 2002), and *Botrytis cinerea* on rose (Morandi *et al.*, 2001). Several known mechanisms employed by the soil-dwelling saprophytic *C. rosea* has been proposed including mycoparasitism, substrate/nutrient competition, enzymatic activity and induced resistance in host plants (Lübeck *et al.*, 2002). The NOI experiments performed in the present Chapter thus provide additional data that support the substrate/nutrient competition hypothesis and might shed light on BCA1's antagonistic effects on FV1 on maize. In the present study, the a_w was modified with PEG 600. In reality, there are a number of other compounds (*e.g.*, sugar alcohols; phenolic compounds; sugars) that can contribute to the modification of a_w and hence different preference/mechanisms could be adopted by individual microorganisms. Therefore, while the *in vitro* results obtained in the present work may serve as an indicator of the possible mechanism of action, more

detailed analyses on the effect of molecular mass of C-sources, and also the effect of other a_w modification solutes may be useful to try and understand the *in situ* biocontrol mechanisms.

To better understand the detailed utilization patterns of C-sources by the antagonist and the pathogen, the TCUS was determined using the Bioscreen-C®. In terms of C-source utilization by FV1, although the percentages of amino acids utilized were higher than that of carbohydrates in all treatments, the TTD for carbohydrates was in fact shorter when compared to amino acids in all treatments. Even though the molecular weights for carbohydrates (180-500 mw; variable for polysaccharides) in the present work are more than double that of amino acids (90-165 mw), the preferential utilization of carbohydrates observed over amino acids in terms of TTD might be explained by the fact that carbohydrates are the primary sources for energy/fuel required for cellular metabolism in almost all organisms. Amino acids also contribute to cellular energy metabolism especially when the primary source of energy is scarce, or when cells undergo metabolic stress (Hothersall and Ahmed, 2013). Similar behaviour was also observed in *Aspergillus flavus* strains and *F. verticillioides* ITEM 1744 (Giorni *et al.*, 2009), as well as in *Penicillium* spp. and *A. ochraceus* (Arroyo *et al.*, 2008). As for the starch C-sources, the longer TTD for amylose as compared to amylopectin might be explained by the fact that by having few α-(1→6) glycosidic bonds, amylose usually takes the form of a long chain which is difficult to hydrolyze and hence longer TTD were observed when compared to amylopectin which has short but highly branched chains [having both α-(1→4) and α-(1→6) bonds]. Therefore, amylopectin is easier to degrade as it has many end points where enzymes can attach (Birt *et al.*, 2013). In

addition, amylopectin, which represents about 70-80% of kernel dry matter, is relevant with regard to fumonisins production (Bluhm and Woloshuk, 2005). The preference in glutamic and aspartic acids might be explained by the fact that these amino acid sources have also been shown to be essential in fumonisin production (ApSimon, 2001) and mycelial formation in several strains of the *Giberella fujikuroi* complex (*F. verticillioides*, *F. proliferatum*) as demonstrated by Jiménez *et al.* (2003).

For BCA1, apart from several similarities to FV1 observed (*i.e.*, carbohydrates preferred over amino acids, amylopectin preferred over amylose), TCUS revealed slightly different patterns of C-source utilization. In contrast to FV1, the majority of the carbohydrate sources (70%) were preferentially utilized by BCA1 at 0.95 a_w when compared to 0.98 a_w. However, the utilization of dextrin under all treatment regimes by the antagonist, with none being utilized by the pathogen shows a clear difference between them as both were able to assimilate amylose and amylopectine (albeit at different rates under different ecophysiological conditions). The relative preference for monosaccharides and disaccharides observed might be explained by the effects of water stress. Under low water stress conditions, disaccharides were preferred by BCA1. As water stress was imposed, the easier-to-degrade monosaccharide was utilized in addition to the disaccharide. However, it is also noteworthy that the different growth rates between the two competing species in the present work might indirectly give rise to the results obtained, and that the zero minute (0 min) in TTD should not be construed as "unutilized" rather it indicated that certain C-sources were perhaps utilized later outside the experimental time frame of seven days (10,080 min). Of course as the antagonist is usually a soil-borne saprophyte and can effectively survive on crop residue it may be

able to compete with *F. verticillioides* via the two modes of infection it utilizes in maize: (1) vertical contamination of surviving spores in the soil that travel up the roots to the foliar plant parts and (2) horizontal contamination of spores by splash dispersal and infection by bird/insects damage.

The findings in the present work contradict those of Mohale *et al*. (2013) who found that irrespective of temperature, at higher a_w levels (0.96, 0.99 a_w), both toxigenic and atoxigenic *A. flavus* strains co-existed. However, this might be explained by the fact that different species might adopt different mechanisms of action under the effects of different ecophysiological conditions which in turn contribute to the different exclusion competence. Furthermore, the present work only used similar concentrations of C-sources as described by Wilson and Lindow (1994). The actual amount of each C-source in maize kernels might be different and this may further influence the utilization patterns (Lee and Magan, 1999; Mohale *et al.*, 2013).

4.5 CONCLUSIONS

Overall, the C-source utilization patterns, NOI and TCUS studies carried out in the present work provided additional information on the nutritional utilization patterns of both the antagonist and the pathogen. The ability of the two competing species to utilize different C-sources demonstrated in the present work reflects their competitiveness under different ecophysiological conditions. By occupying similar niches under several ecophysiological conditions, *C. rosea* 016 (BCA1) appeared to have antagonistic properties which may provide an ecological advantage in controlling growth of *F. verticillioides* and reducing fumonisin production. In the present study the bacterial

candidate (BCA5) was unable to grow in any of the treatments and could not thus be included in subsequent studies. This may have been due to the solute used (PEG 600) which could be toxic to some bacteria (Kinnunen and Koskela, 1991; Cox, 1966).

CHAPTER 5

Effects of biocontrol agents in controlling fumonisin B_1 production by Fusarium verticillioides FV1 in maize cobs of different ripening stages by monitoring both FUM1 gene expression and phenotypic toxin production

5.1 INTRODUCTION

The fumonisin biosynthetic genes (*FUM* genes) are clustered together (Proctor *et al*., 2003). One of the cluster genes, *FUM1*, encodes for a polyketide synthase (PKS) which is required for fumonisin biosynthesis (Proctor *et al*., 1999). In effect, this PKS is the catalyst in the initial step for fumonisin biosynthesis (Bojja *et al*., 2004). Extensive studies have been performed on expression of *FUM* genes *in vitro* (Jiménez *et al.*, 2003; López-Errasquín *et al.*, 2007; Jurado *et al.*, 2008; Lazzaro *et al.*, 2012b, c; Medina *et al.*, 2013). However, the expression of the *FUM* genes on maize cobs (*in planta*) by artificial inoculation has surprisingly not been examined previously. In this Chapter, the efficacy of the two best biocontrol agent (BCA) candidates have been tested for control of FB_1 production on maize cobs of different ripening stages by measuring both *FUM1* gene expression and toxin contamination.

Therefore, the objectives of this Chapter were:

(a) To study the effects of incubation periods on *FUM1* gene expression and FB_1 production by *Fusarium verticillioides* FV1 on maize kernels

(b) To examine the effects of artificial point inoculation of antagonists and pathogen (50:50 ratio) on maize cobs at three different ripening stages and thus a_w levels, on *FUM1* gene expression using q-PCR and FB_1 production using HPLC-FLD

5.2 MATERIALS AND METHODS

5.2.1 Microorganisms

A fumonisin-producing strain of *Fusarium verticillioides* FV1 which was isolated from Malaysian maize kernels and identified morphologically and molecularly as described in Subsection 2.2.5 was used. BCA1 (*Clonostachys rosea* 016) and BCA5 (Gram-negative bacterium) which exhibited inhibition in FB_1 production in maize agar and maize kernels in Chapter 3 were maintained at 25°C on Potato Dextrose agar and Nutrient agar respectively, and used to examine biocontrol of the pathogen on maize cobs.

5.2.2 Sampling of maize cobs of different ripening stages

Maize cobs of different ripening stages were obtained at three reproductive stages (R_3 milk, R_4 dough, and R_5 dent stages) from the NIAB Institute farm (National Institute of Agriculture and Botany, Cambridge, UK) in September and October 2012. The type of maize was ES Regain (Euralis Semences; a type of forage maize). Stratified sampling was employed in which the maize cobs were taken from a number of subpopulations (*i.e.*, entrance, middle, exit of plot) within a field to ensure standard segregation of nutrient in soil. Harvested maize cobs were promptly taken back to the laboratory where the a_w was measured for sub-samples of detached kernels as detailed previously in Subsection 2.2.2. To ensure a representative reading of a_w from the entire cob, five to ten maize kernels were taken from the top, middle and bottom parts of the cobs (Table 5.1). All the other maize cobs from the three ripening stages were then flash-frozen by submerging the cobs into liquid Nitrogen for five to ten seconds so that large ice

crystals from intra- and extracellular water did not form and damage the sample (*i.e.*, cell membranes) during storage at -4°C.

Table 5.1. Maize cobs of different ripening stages (R), and points for measurement of water activity.

REPRODUCTIVE GROWTH STAGE	IMAGE
R_3: milk • Rapid grain filling period. • Kernels begin to show yellowish colour. • Fluid filling the kernels has a milky texture, and is white in colour.	
R_4: dough • Kernels begin to firm up and show a brighter yellow colour. • Starch in kernels begins to harden (decrease in liquid, increase in solid) into a doughy consistency.	
R_5: dent • Kernels are denting or already dented with reddish colouration. • Kernels have a harder texture than dough stage.	

5.2.3 Effects of incubation periods and water activities on *FUM1* gene expression and FB_1 production by *Fusarium verticillioides* FV1 on maize kernels

Ten gram dry gamma-irradiated maize kernels were aseptically transferred into 55 mm Ø Petri plates. The a_w of the dry maize kernels were modified to 0.955 (0.95 a_w) and 0.982 (0.98 a_w) by reference to the moisture adsorption curve for maize kernels

described in Subsection 3.2.4. The spore inoculum of FV1 was obtained according to procedures described in Subsection 3.2.5. The amount of sterilized water added for each treatment is tabulated in Table 5.2. After 24 hours equilibration period, the layers of maize were aseptically inoculated throughout the plates with 1 mL spore inoculum at a concentration of $\approx 10^6$ spore/mL. All experiments were carried out with three replicates.

Table 5.2. Amount of sterilized water and spore suspension added to the 10 g dry maize kernels to modify the water activity.

Water activity (a_w)	Sterilized water (mL)	Spore inoculum (mL)	Total (mL)
0.98	3.5	1.0	4.5
0.95	1.5	1.0	2.5

Treatments were incubated at 25°C in separate polyethylene environmental chambers each containing a beaker of glycerol/water solution to help maintain the ERH of the atmosphere as the target a_w levels of the experiment. After 5, 7, 10 and 14 days incubation, Petri plates containing mouldy maize kernels were destructively sampled with half being used for molecular analyses and half for FB_1 quantification. The maize samples for molecular work were immediately frozen in liquid Nitrogen and stored at -80°C until analyses was done.

(a) *FUM1* gene expression

For *FUM1* gene expression analysis, four sequential steps were carried out: (a) RNA isolation of *F. verticillioides* FV1 from maize kernels, (b) reverse transcription to convert mRNA into complementary DNA (cDNA), (c) amplification of *FUM1* gene

using real-time quantitative PCR (q-PCR), and (d) absolute quantification of *FUM1* gene expression using the standard curve method.

(i) RNA isolation of FV1 from maize kernels

RNeasy® Plant Mini Kit (Qiagen, Germany) was used for RNA isolation. Manufacturer's instructions were followed. An amount of 2-3 mouldy maize kernels was placed into an extraction tube and 1 mL of RLT buffer (the lysis buffer provided by RNeasy® Plant Mini Kit, supplemented with β-mercaptoethanol) was added and vortexed vigorously for 10 seconds. The lysate was then transferred into a QIAshredder spin column placed in a 2 mL collection tube and centrifuged (Biofuge, Germany) for two minutes at 10,000 rpm. The supernatant was carefully transferred into a new microcentrifuge tube without disturbing the cell-debris pellet. Half a volume of absolute ethanol (96-100%) was added to the cleared supernatant (≈700 µL), and immediately mixed by pipetting. The supernatant-ethanol solution was transferred into an RNeasy® Mini spin column placed in a 2 mL collection tube and centrifuged for 15 seconds at 10,000 rpm and the flow-through was discarded. Next, 700 µL of RW1 buffer (the washing buffer provided) was added into the RNeasy® Mini spin column and centrifuged for 15 seconds at 10,000 rpm and the flow-through was discarded. Then, 500 µL RPE Buffer (the precipitation buffer provided, supplemented with ethanol) was added into the RNeasy® Mini spin column and centrifuged for 15 seconds at 10,000 rpm and the flow-through was discarded. The latter step was repeated twice. Next, RNeasy® Mini spin column was centrifuged for two minutes at 10,000 rpm to ensure that all ethanol was dried to avoid RNA denaturation. The RNeasy® Mini spin column was placed into a new 1.5 mL collection tube and 50 µL RNase-free water was added

into the RNeasy® Mini spin column before centrifuging it for one minute at 10,000 rpm to elute the RNA. The eluted RNA was stored at -80°C until further analysis. The RNA concentration and purity (A_{260}/A_{280} ratio) were determined spectrophotometrically using a 2.5 µL aliquot on the Picodrop™ (Spectra Services Inc., USA). A ratio between 1.8 and 2.1 is indicative of highly purified RNA (Ahmad-Ganaie and Ali, 2014; Gallagher, 2001).

(ii) Reverse transcription to convert mRNA into cDNA

The Omniscript® RT Kit (Qiagen, Germany) was used to perform the reverse transcription for first-strand cDNA (complementary) with RNA as the starting template (cDNA synthesis). The kit contained Omniscript® Reverse Transcriptase, 10x buffer RT; dNTPs (deoxynucleoside triphosphates) mix (5 mM each) and RNase-free water. The oligo-dT primer (a short sequence of deoxy-thymine nucleotides) was purchased separately (Qiagen, Germany). Manufacturer's instructions were followed. First, the RNA templates which were purified earlier were thawed on ice, and all the buffer and reagents were brought to ambient temperature. Each solution was homogenized by vortexing. Then, a master mix solution was prepared according to Table 5.3, mixed thoroughly and carefully by vortexing for no more than five seconds, and stored on ice. The tubes were labelled according to the RNA templates. Next, 15 µL aliquots of master mix solution were dispensed into the PCR tubes before adding 5 µL total RNA template into each of the tube using filtered pipette tips (in total, 20 µL per reaction tube). A thermal cycler (Techne™ Thermal cycler TC-312, UK) was used to complete the reaction for 60 minutes at 37°C followed by five minutes at 93°C. The resulting cDNA were stored at -20°C until further use.

Table 5.3. Master mix components for reverse transcription per reaction.

COMPONENT	VOLUME
Buffer RT, 10x	2 µL
dNTPs mix	2 µL
Oligo-dT primer	2 µL
Omniscript® Reverse Transcriptase	1 µL
RNase-free water	8 µL
TOTAL	**15 L**

(iii) Amplification of FUM1 gene using real-time quantitative PCR (q-PCR)

A real-time quantitative PCR (q-PCR) was performed to amplify and quantify the *FUM1* gene. The non-specific intercalating dye SYBR® Green was used to detect target amplification since it is an easier and cheaper alternative to the specific hydrolysis probes. Primer design and optimization of qPCR conditions were performed before conducting the assay. In SYBR® Green assays, the proper design of primers is critical as the dye intercalates into double-stranded DNA without distinguishing between specific and nonspecific q-PCR products (Rodríguez *et al.*, 2015). In this work, the primers PQF1-F (5'-GAGCCGAGTCAGCAAGGATT-3') and PQF1-R (5'-AGGGTTCGTGAGCCAAGGA-3'; López-Errasquín *et al.*, 2007) were used. The concentration of primers and reagents were optimized by selecting the combination which gave the lowest value of quantification cycle (C_q; the cycle at which the fluorescent signal crosses the threshold line or exceeds background; used interchangeably with C_t) in the amplification plots, and the highest fluorescent signal for a fixed target concentration. Optimal cycling conditions were determined by testing several annealing temperatures, starting around 5°C below the T_m (temperature at which

half of the double-strand DNA are in single-strand form). List of crucial parameters to be considered when designing primers and probes for real-time quantitative PCR is tabulated in Appendix K.

To perform the q-PCR, the cDNA templates were thawed and placed on ice. The primer solutions, SYBR® Green dye and RNase-free water were also thawed and mixed thoroughly by inverting the tubes several times to ensure homogeneity before placing them on ice. The light-sensitive SYBR® Green dye tube was covered at all time. A 10-fold dilution was then made for each of the cDNA templates (10 µL cDNA in 90 µL RNase-free water). A fresh master mix was prepared according to Table 5.4 before aliquoting 10 µL into individual q-PCR tubes (Bio-Rad, USA). Next, 2.5 µL of each cDNA templates was carefully added into the individual wells containing the master mix in triplicate, and mixed thoroughly but gently. In all the experiments, appropriate negative controls containing no cDNA template were subjected to similar procedure to exclude or detect any possible contamination or carryover (López-Errasquín *et al.* 2007). Each triplicate was amplified in two tubes (a total of six measurements per sample). The cycling conditions were set according to Table 5.5. The CFXConnect™ Real-Time System (Bio-Rad, USA) was used to perform the q-PCR assay, and the C_q values were recorded by the Bio-Rad CFX Manager Version 3.1 software (Bio-Rad, USA).

(iv) Absolute quantification of FUM1 gene expression using the standard curve method

Absolute quantification of *FUM1* gene expression using the standard curve method was carried out according to Livak and Schmittgen (2001) with the C_q values provided by the software. The standard curve was constructed to estimate the efficiency of the designed method by plotting the C_q values on the *y*-axis against a series (usually five) of increasing and known concentrations of the RNA template (10-fold serial dilutions) on the *x*-axis. The *x*-axis can also be weight (*e.g.*, ng), concentration (*e.g.*, ng/µL) or number of gene copies (usually in the form of $\log_{10}$). Figure 5.1 illustrates the standard curve for the present work. Since it takes 3.32 cycles for a 100%-efficient PCR amplification to increase the number of template molecules to 10-fold ($2^{3.32} = 10$) in any given sample, the efficiency of the designed q-PCR can be checked by; efficiency = $10^{(-1/\text{slope})} - 1$. In the present work, the slope is -3.3505 which equals to 98.82% efficiency. Usually, slopes between -3.1 and -3.6 with q-PCR efficiency values in the range of 90-110% are considered satisfactory provided that the correlation coefficient (R^2) derived from the standard curve is between 0.98 and 0.99.

Table 5.4. Master mix components for q-PCR per reaction.

COMPONENT	VOLUME
SYBR® Green dye, 2x	6.25 µL
10 µM forward primer	0.375 µL
10 µM reverse primer	0.375 µL
RNase-free water	3 µL
TOTAL	**10 µL**

Table 5.5. Optimized cycling conditions for q-PCR.

STEP	TEMPERATURE	TIME	NO. OF CYCLE
Enzyme activation	95ºC	30 sec	1
Denaturation	95ºC	5 sec	30-40
Annealing	55 - 60ºC	20 sec	
Melting curve	65 - 95ºC	5 sec	1

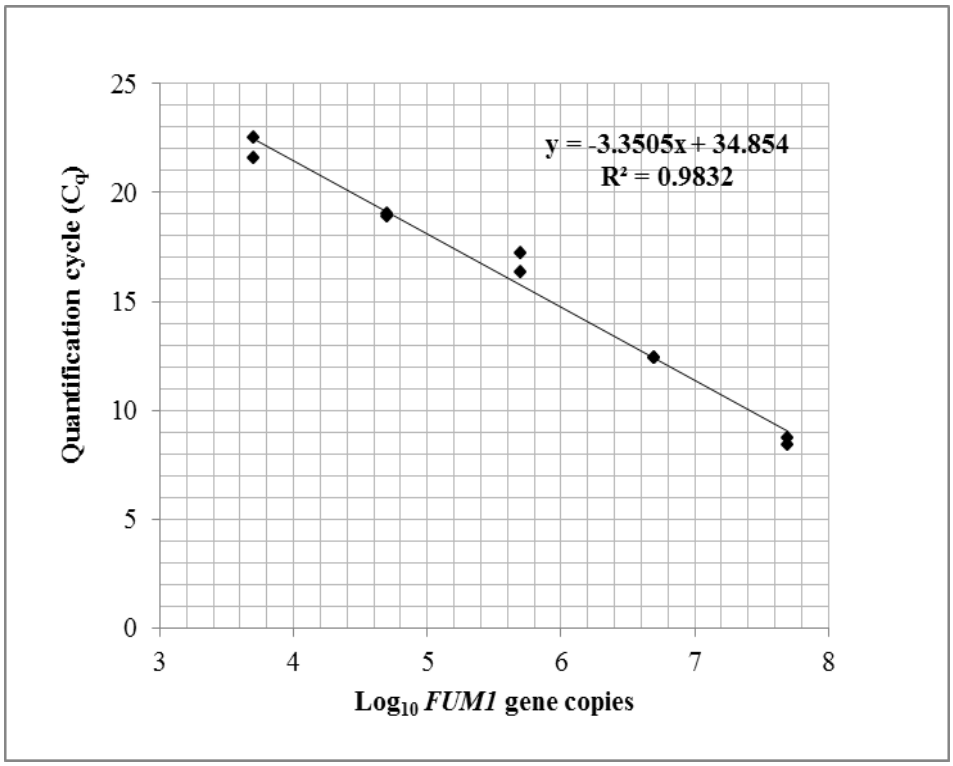

Figure 5.1. Standard curve for the absolute quantification of *FUM1* gene expression.

(b) Fumonisin B_1 quantification

Solvent extraction for FB_1 from the maize kernels, and the subsequent clean-up using the FumoniStar™ Immunoaffinity Columns (Item No. COIAC3000; Romer, Austria), were carried out according to procedures described in Subsection 4.2.4. Separation and

detection of FB_1 by a reversed phase-HPLC system joined to a fluorescence detector (HPLC-FLD) were performed according to procedures described in Subsection 4.2.5. Recovery rate (%) was obtained within the range of manufacturer's specification by spiking a known concentration of FB_1 and compared with the results of HPLC-FLD quantification.

5.2.4 Effects of biocontrol agents (BCAs) on *FUM1* expression and FB_1 production by *Fusarium verticillioides* FV1 on maize cobs of different ripening stages

Flash-frozen maize cobs (R_3, Milk; R_4, Dough; and R_5, Dent) were thawed and divided into two batches for the two temperature treatments used; 25°C and 30°C. Separate environmental chambers were used for each treatment and set of replicates. The treatments were: *F. verticillioides* FV1 alone (negative control); FV1 + BCA1 (*Clonostachys rosea* 016) and FV1 + BCA5 (gram-negative motile bacterium). These were used as they exhibited best control on FB_1 production in maize-based media and on stored maize kernel assays (Chapter 3).

Spore suspensions were made up to achieve $\approx 10^6$ spores/mL concentration for FV1, BCA1, and BCA5 according to procedures described in Subsection 4.2.3. Three replicate maize cobs were inoculated at three points with 100 µL spore suspension of FV1 (negative control), 100 µL FV1 + 100 µL BCA1, and 100 µL FV1 + 100 µL BCA5, and placed in each of the treatment conditions. A beaker containing glycerol/water solution was included in the chamber to help maintain the ERH at the target a_w of maize cob treatments. Figure 5.2 illustrates the experimental design. All

containers containing inoculated maize cobs were incubated for ten days. At the end of the incubation, 10-15 mouldy kernels were removed, labelled, and divided into two equal batches for *FUM1* gene expression studies by real time q-PCR and FB_1 toxin production studies by HPLC-FLD. The q-PCR was performed according to procedures described in Subsection 5.2.3, and the HPLC-FLD was performed according to procedures described in Subsection 4.2.5.

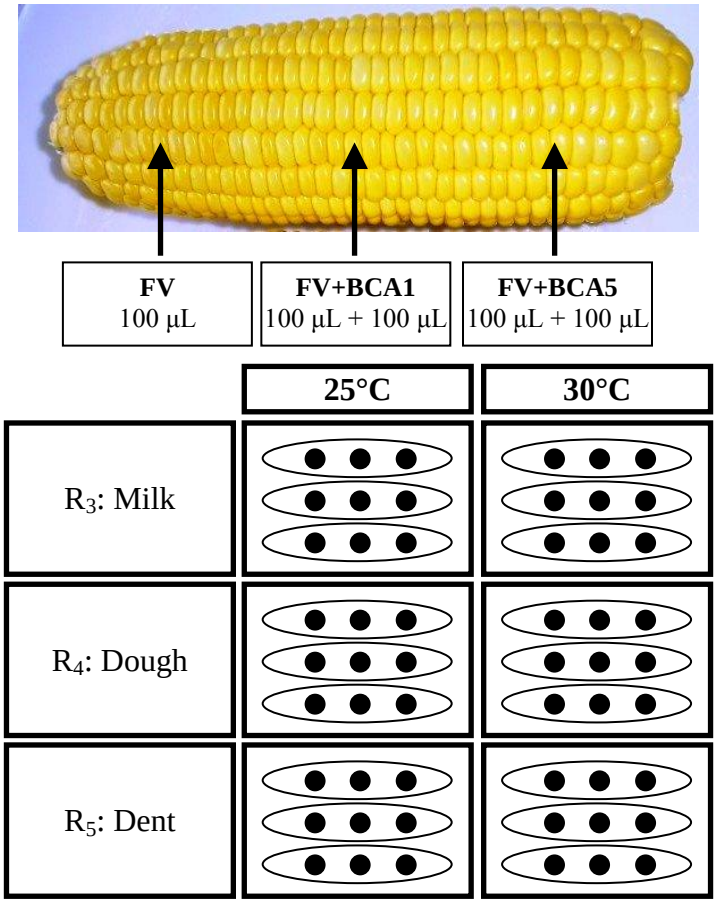

Figure 5.2. Experimental design for the effects of BCAs on *FUM1* expression and FB_1 production by *Fusarium verticillioides* FV1 on maize cobs of different ripening stages.

5.2.5 Statistical analysis

All experiments were carried out in three replicates per treatment. Measurements were then averaged and presented as mean ± SE (standard error). Normal distribution of datasets was checked by the Kolmogorov-Smirnov normality test. Analysis of Variance (ANOVA) was applied on normally distributed datasets to analyse the variation between and within group means with 95% confidence interval. $p < 0.05$ was accepted as significant difference. Fisher's Least Significant Difference (LSD) with $\alpha = 0.05$ was applied to compare significant difference between means of treatments using the statistical software Minitab® version 14.0 (Minitab Inc.; Pennsylvania, USA).

Figure 5.3 summarizes the key experimental steps carried out in this Chapter.

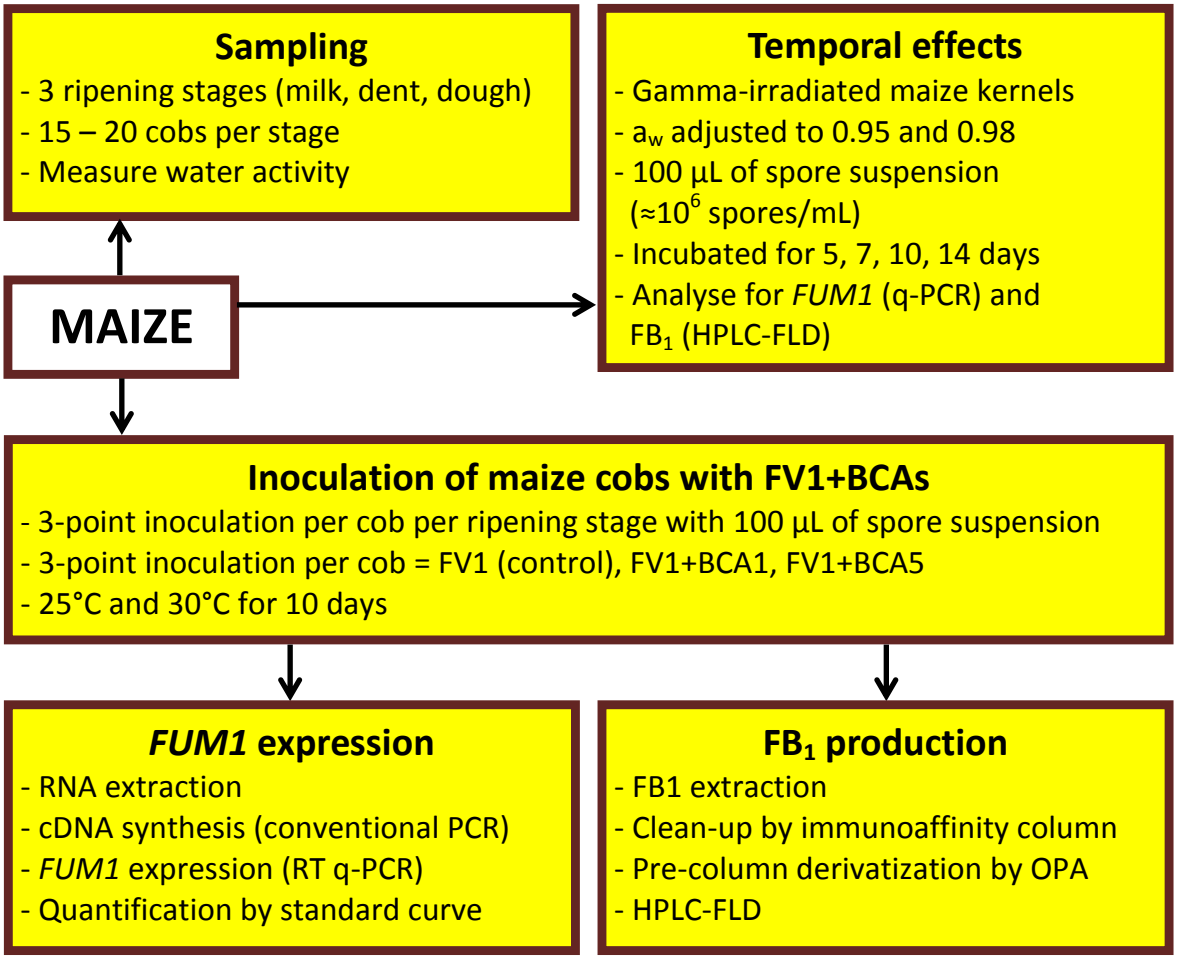

Figure 5.3. Key experimental steps carried out in Chapter 5 "Effects of biocontrol agents in controlling fumonisin B_1 production by *Fusarium verticillioides* FV1 in maize cobs of different ripening stages by monitoring both *FUM1* gene expression and phenotypic toxin production".

5.3 RESULTS

5.3.1 Water activity of maize cobs of different ripening stages

Figure 5.4 shows the a_w of maize cobs of different ripening stages. It was apparent that as the maize neared maturity, the a_w of the kernels was significantly decreased ($p < 0.05$) from R_3, Milk (0.985 ± 0.001 a_w) to R_4, Dough (0.976 ± 0.002 a_w) and to R_5, Dent (0.958 ± 0.002 a_w).

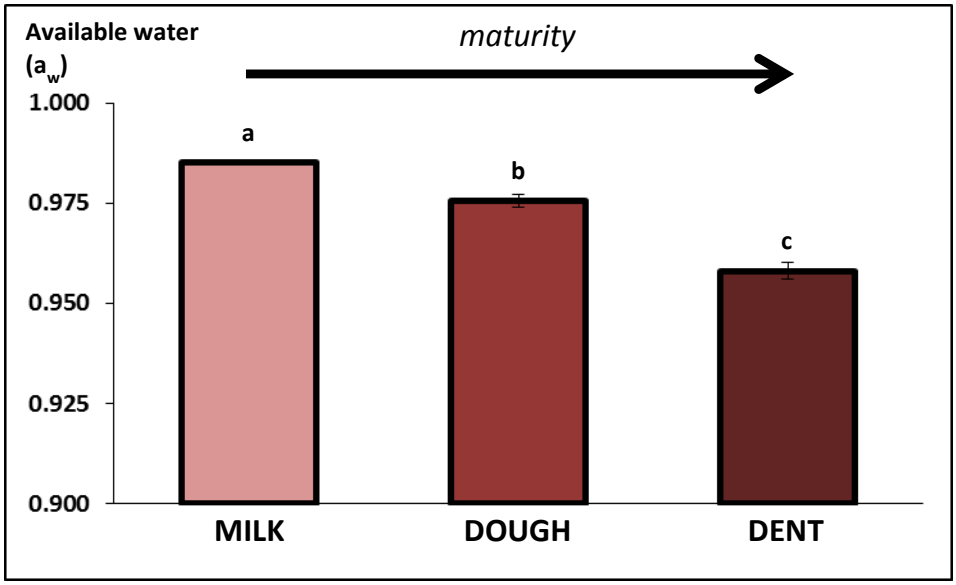

Figure 5.4. Water activity of maize cobs of different ripening stages. Data are means of 12 readings (n = 12) with bars indicating standard error. Different letters indicate significant difference ($p < 0.05$) by Fisher's Least Significant Difference (LSD).

5.3.2 Effect of incubation periods and water activities on *FUM1* gene expression and FB_1 production by *Fusarium verticillioides* FV1 on maize kernels

The effect of a_w and incubation time on *FUM1* gene expression on the maize kernels inoculated with FV1 is shown in Figure 5.5. At 0.95 a_w, there was a significant stimulation in *FUM1* gene expression after 10 and 14 days ($p < 0.05$). However, at 0.98

a_w, there was no significant increase in *FUM1* gene expression at any of the sampling times evaluated. The *FUM1* gene expression was higher at 0.98 a_w than 0.95 a_w at all the sampling times.

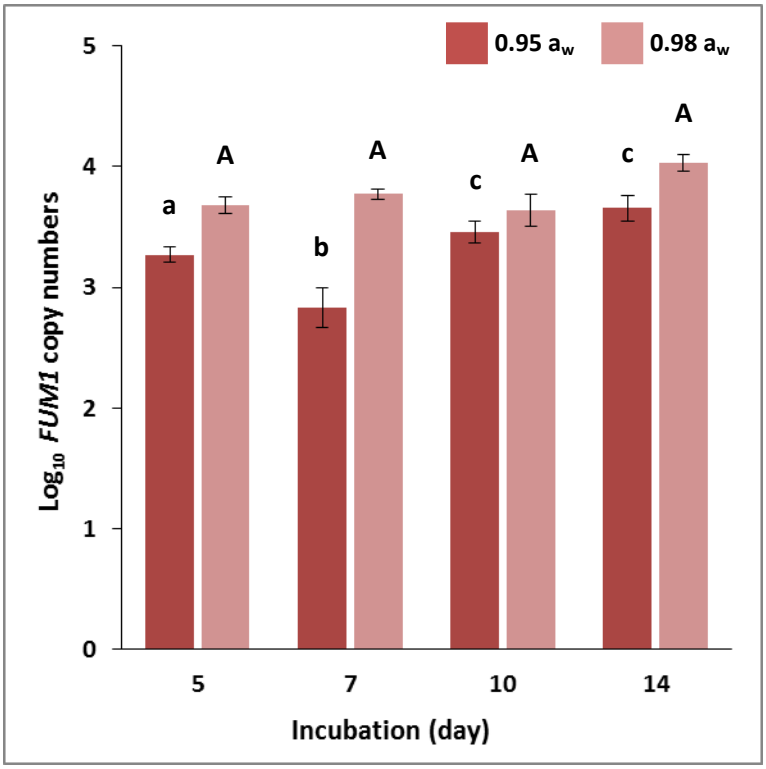

Figure 5.5. *FUM1* gene expression by *Fusarium verticillioides* FV1 on the maize kernels at 0.95 and 0.98 a_w after 5, 7, 10 and 14 days incubation at 25°C. Data are means of triplicates (n = 3) with bars indicating standard error. Different letters indicate significant difference (p < 0.05) by Fisher's Least Significant Difference (LSD). Amplification cycle = 40. Log_{10} 1 = 10 copy numbers.

Figure 5.6 shows the effect of a_w and incubation time on FB_1 production by FV1 on the maize kernels. There was a higher production of FB_1 at 0.95 a_w when compared to 0.98 a_w at all sampling times (p < 0.05). At 0.95 a_w, FB_1 production was highest after 5 and 10 days incubation. However, at 0.98 a_w, this was highest after 7 and 14 days (p < 0.05).

Images of temporal colonization of FV1 on the layers of maize kernels at 0.95 and 0.98 a_w are shown in Appendix L.

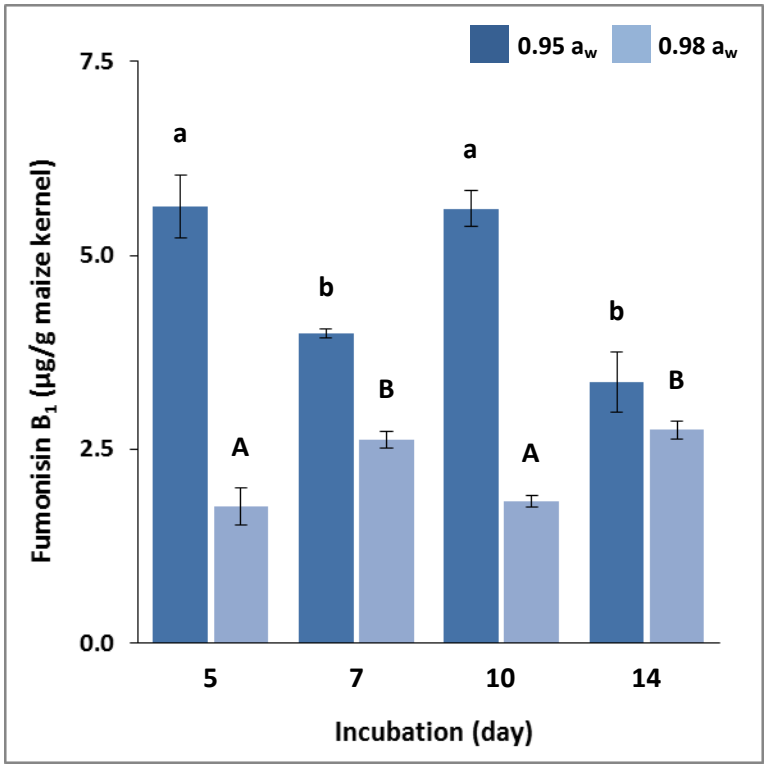

Figure 5.6. Fumonisin B_1 production by *Fusarium verticillioides* FV1 on the maize kernels at 0.95 and 0.98 a_w after 5, 7, 10 and 14 days of incubation at 25°C. Data are means of triplicates (n = 3) with bars indicating standard error. Different letters indicate significant difference ($p < 0.05$) by Fisher's Least Significant Difference (LSD).

Significant interactions ($p < 0.05$) between the effects of a_w levels and incubation time on *FUM1* gene expression and FB_1 production were obtained (Table 5.6).

Table 5.6. *P*-values for the effects of water activity and incubation time, and their interactions on *FUM1* gene expression (log_{10} copy number) and fumonisin B_1 production (µg/g maize kernel) as analysed by Analysis of Variance (ANOVA).

		FUM1	**FB_1**
Source of variation	**df[1]**	***p*-value**	
Between groups[2] (water activity)	1	0.0000*	0.0000*
Within groups[3] (incubation interval)	3	0.0000*	0.0287*
Interaction	3	0.0038*	0.0000*

[1] Degrees of freedom. [2] Number of water activity (2) minus 1. [3] Number of incubation interval (4) minus 1. * Significant at p < 0.05.

Positive correlations (Pearson's correlation coefficient, *r*) were also obtained between *FUM1* gene expression and FB_1 production at different a_w levels and incubation times (Table 5.7).

Table 5.7. Pearson's correlation coefficient (*r*) for the correlation between *FUM1* gene expression (log_{10} copy number) and fumonisin B_1 production (µg/g maize kernel) at different water activity and incubation interval.

	0.95 a_w	**0.98 a_w**
Day 5	0.9449	0.9256
Day 7	0.9439	0.6617
Day 10	0.9635	0.7274
Day 14	0.7010	0.9097

5.3.3 Effects of biocontrol agents (BCAs) on *FUM1* expression and FB_1 production by *Fusarium verticillioides* FV1 on maize cobs of different ripening stages

Figure 5.7 shows the colonization of maize cobs at R_5 ripening stage after 10 days incubation at 25°C. In general, at all temperatures and ripening stages tested, treatments FV1 (control) and FV1+BCA5 have a similar colony appearance of FV1 (cottony, salmon-coloured). However, the FV1+BCA1 in contrast, predominantly exhibited the colony appearance of BCA1 (velvety, white-coloured). Full images of colonization from point inoculations of the maize cobs at different ripening stages with the mixture and control are shown in Appendix M.

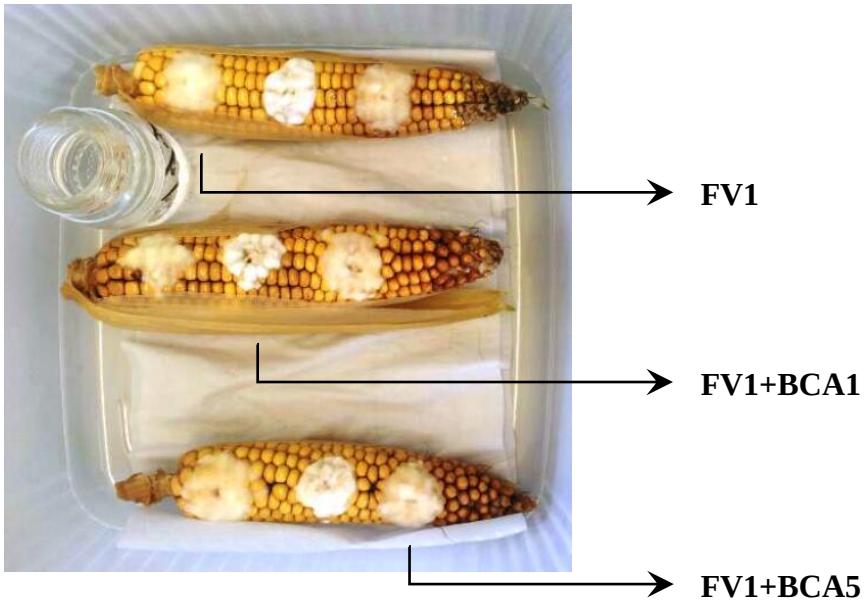

Figure 5.7. Development of fungal colonization of R_5 maize cobs after 10 days incubation at 25°C.

Figure 5.8 compares the diametric colony development (cm) of FV1, FV1+BCA1 and FV1+BCA5 on maize cobs (*in planta*) of different ripening stages (R_3, R_4, R_5) after 10

days incubation at both 25 and 30°C. There were no significant differences ($p > 0.05$) between treatments and at different ripening stages at 25°C. However, at 30°C, no growth was observed at the R_3 ripening stage while sparse growths were observed at the R_4 ripening stage. At R_4 and R_5 ripening stages, the colony diameters of all treatments appeared to be significantly ($p < 0.05$) smaller at 30°C when compared to 25°C.

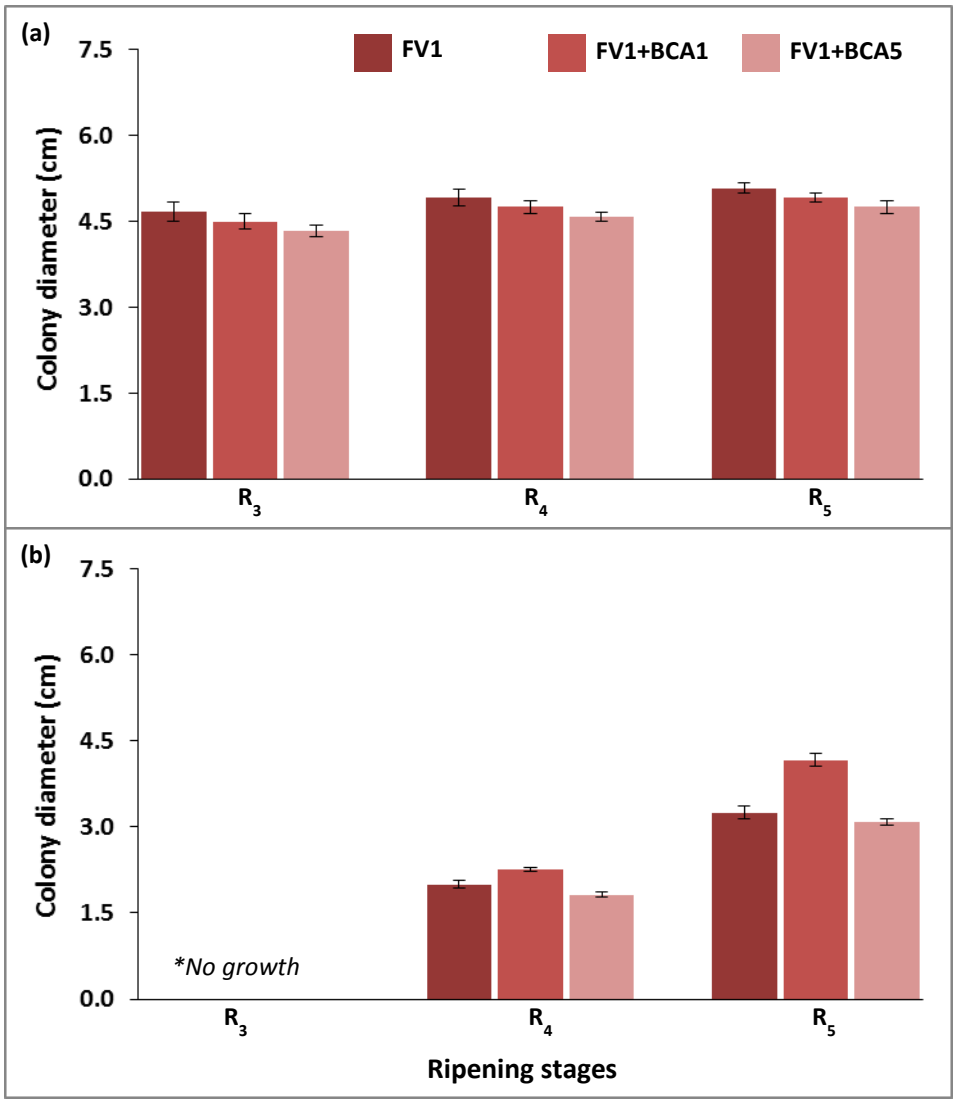

Figure 5.8. Diametric colonization (cm) by FV1, FV1+BCA1 and FV1+BCA5 on maize cobs of different ripening stages (R_3, R_4, R_5) after 10 days incubation at (a) 25°C and (b) 30°C. Data are means of triplicates ($n = 3$) with bars indicating standard error.

Figure 5.9 shows the effect of treatments at different ripening stages (R_3, R_4, R_5) on *FUM1* gene expression after 10 days incubation at 25 and 30°C. At both temperatures tested, across the different ripening stages, there were no significant differences ($p > 0.05$) between FV1 and FV1+BCA5 treatments. However, in the FV1+BCA1 there were significant differences ($p < 0.05$) when compared to the control (FV1) at both temperatures. A significant difference ($p < 0.05$) was also observed in the FV1+BCA1 treatments between the R_4 and R_5 ripening stages at both temperatures.

Figure 5.10 shows the FB_1 production by the different treatments at each of the ripening stages (R_3, R_4, R_5) after 10 days incubation at 25 and 30°C. Overall, the control treatments (FV1) had the highest FB_1 levels at all ripening stages and temperatures tested. The mixture of FV1 and either BCA1 or BCA5 showed significant differences ($p < 0.05$) when compared to the control at both temperatures tested. It is also noteworthy that while both BCAs were significantly different ($p < 0.05$) from each other at the R_5 ripening stage, both treatments showed no significant difference ($p > 0.05$) at the R_4 stage at both temperatures tested. In the control treatments at both temperatures, an increase of FB_1 occurred in all the ripening stages.

Table 5.8 shows the statistical analyses of temperatures and cob ripening stages and their interactions on *FUM1* gene expression and FB_1 production.

Table 5.9 shows the positive correlations (Pearson's correlation coefficient, r) between *FUM1* gene expression and FB_1 production at different temperatures and cob ripening stages.

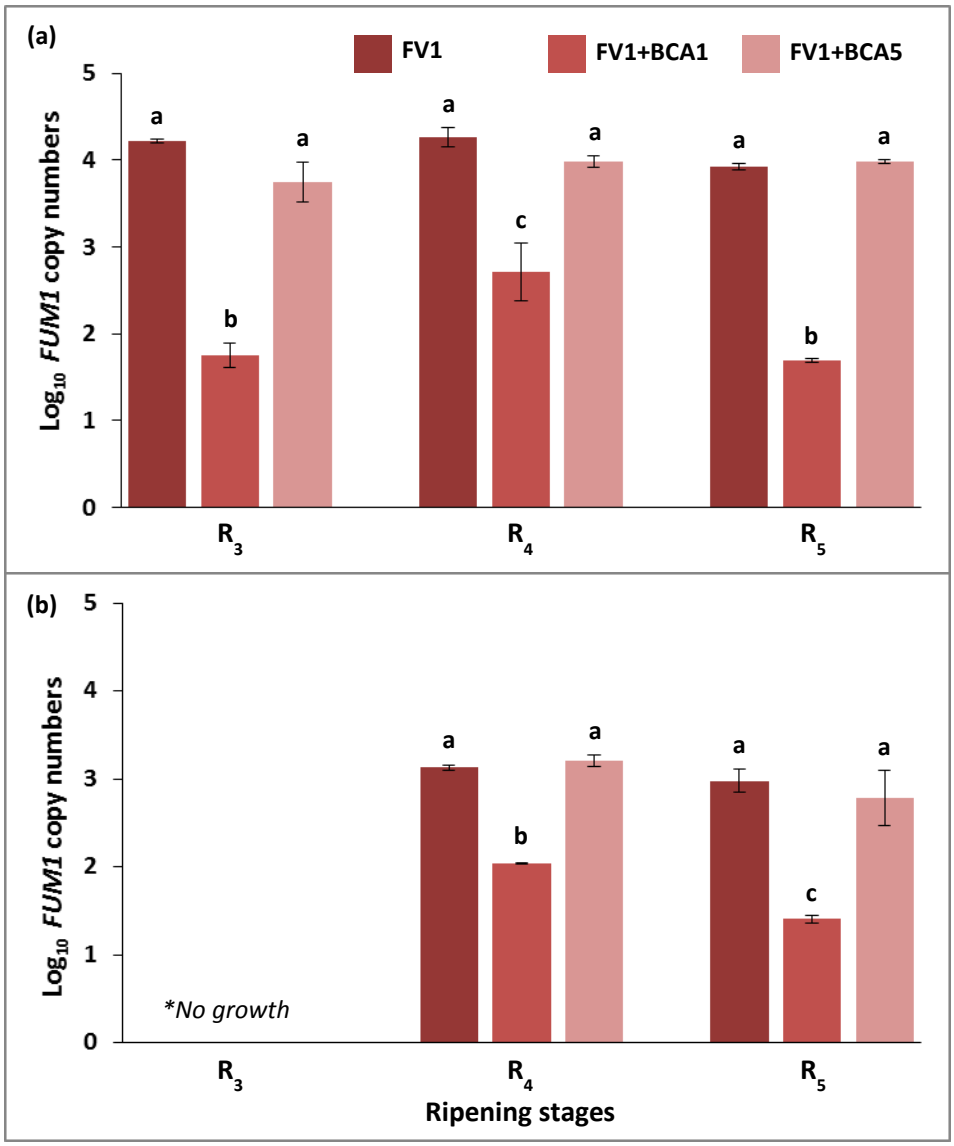

Figure 5.9. *FUM1* gene expression by FV1, FV1+BCA1 and FV1+BCA5 on maize cobs of different ripening stages (R_3, R_4, R_5) after 10 days incubation at (a) 25°C and (b) 30°C. Data are means of triplicates (n = 3) with bars indicating standard error. Different letters indicate significant difference ($p < 0.05$) by Fisher's Least Significant Difference (LSD). Amplification cycle = 40. Log_{10} 1 = 10 copy numbers.

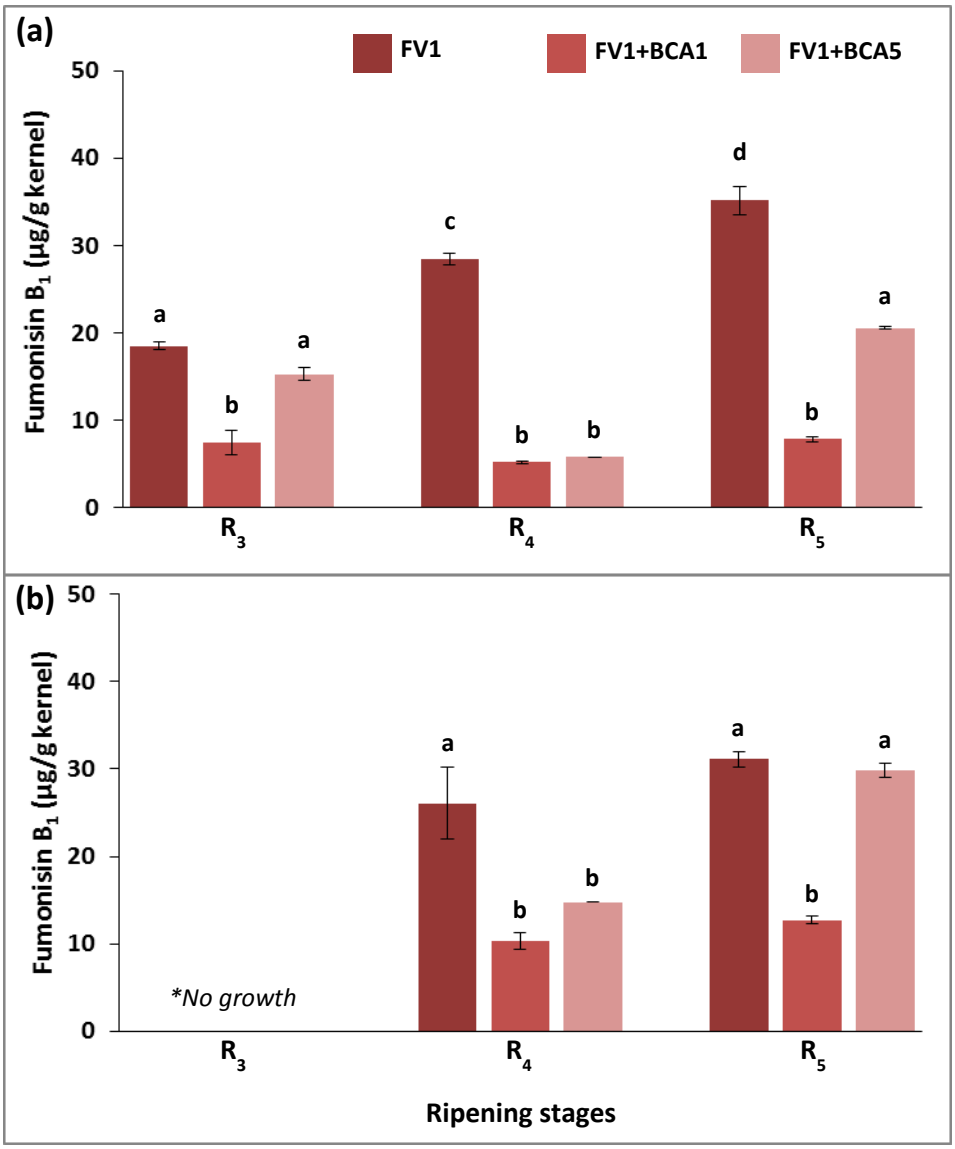

Figure 5.10. Fumonisin B_1 production by FV1, FV1+BCA1 and FV1+BCA5 on maize cobs of different ripening stages (R_3, R_4, R_5) after 10 days incubation at (a) 25°C and (b) 30°C. Data are means of triplicates (n = 3) with bars indicating standard error. Different letters indicate significant difference ($p < 0.05$) by Fisher's Least Significant Difference (LSD).

Table 5.8. *P*-values for the effects of temperature and cob ripening stage, and their interactions on *FUM1* gene expression (log_{10} copy number) and fumonisin B_1 production (µg/g maize kernel) as analysed by Analysis of Variance (ANOVA).

		FUM1	**FB_1**
Source of variation	**df[1]**	***p*-value**	
Between groups[2] (temperature)	1	0.0000*	0.0004*
Within groups[3] (ripening stage)	2	0.0000*	0.0000*
Interaction	2	0.0000*	0.0000*

[1] Degrees of freedom. [2] Number of temperature (2) minus 1. [3] Number of ripening stage (3) minus 1. * Significant at p < 0.05.

Table 5.9. Pearson's correlation coefficient (*r*) for the correlation between *FUM1* gene expression (log_{10} copy number) and fumonisin B_1 production (µg/g maize kernel) at different temperatures and cob ripening stages.

	25°C	**30°C**
R_3	0.8985	**no growth*
R_4	0.7025	0.6766
R_5	0.8608	0.9207

5.4 DISCUSSION

5.4.1 Effects of incubation periods and water activities on *FUM1* gene expression and FB_1 production by *Fusarium verticillioides* FV1 on maize kernels

On irradiated maize kernels, FB_1 production was higher at 0.95 a_w at all sampling times (5, 7, 10, 14 days) tested. Fluctuation in FB_1 levels observed during the time course might be explained by the fact that (1) oxygen was decreased during incubation, (2) the fungus broke down its own toxin and then synthesized it again in a cyclic way (LeBars *et al*. 1994), or (3) due to the enzymatic cleavage of the toxin, or its conversion to other related compounds, or both (Alberts *et al*. 1990). A strong correlation was obtained between *FUM1* gene expression and FB_1 production at 0.95 a_w (Pearson's r = 0.8884 ± 0.1252) and 0.98 a_w (Pearson's r = 0.8061 ± 0.1317) at the different sampling times tested. The findings in the present study are in agreement with that of Marín *et al*. (2004) and Mogensen *et al*. (2009) who previously reported the optimum temperature and a_w for inducing fumonisin production ranged from 20 to 25°C, and 0.95 to 0.99 a_w, while no production observed at ≤ 0.93 a_w and 10°C. A lower optimum temperature of 20°C was also reported by Medina *et al*. (2013) by another strain of *F. verticillioides* on maize-based media. Previous studies have also shown positive correlation between *FUM1* gene expression and fumonisin production (Jurado *et al.* 2008; López-Errasquín *et al.* 2007). It has also been shown that ecophysiological conditions could affect the expression of genes and the subsequent biosynthesis of secondary metabolites (Lazzaro *et al*. 2012b). The fluctuation in *FUM1* gene expression observed might be explained by the differences in the temporal expression patterns at the two a_w levels tested.

Although a strong and positive correlation was found between *FUM1* gene expression and FB_1 production at both a_w levels tested, there was a relative difference in which *FUM1* gene was expressed to higher levels at 0.98 a_w when compared with that at 0.95 a_w whereas FB_1 was produced more at 0.95 a_w than 0.98 a_w. These findings seem to concur with that of Jurado *et al*. (2008). However, the a_w in these *in vitro* studies on a secondary metabolite conducive medium (FIM) was adjusted with solutes such as glycerol, NaCl or PEG 8000, and no correlation analysis with FB_1 production was performed.

The study has focused on the *FUM1* gene because of its importance in the biosynthetic pathway for fumonisin production. However, other genes such as *FUM19* which encodes for an ABC (ATP-Binding Casette) transporter involved in the extracellular export of fumonisins (Proctor *et al*. 2003) may also be important in the regulation of production. Although in 2007, López-Errasquín and co-workers described a strong correlation between the expression of *FUM1* and *FUM19* after three and seven days incubation, the effects of a_w were not tested, and the experiments were done on a fumonisin-inducing growth medium (López-Errasquín *et al.*, 2007). Furthermore, Lazzaro *et al*. (2012c) highlighted the apparent differences in strains' genotypic (gene expression) and phenotypic (secondary metabolite production) characteristics which may due to differences in genetic backgrounds. While Medina *et al*. (2013) examined the expression of the whole cluster of *FUM* genes, and focused on expression of nine genes in the biosynthetic pathway in relation to environmental factors in an attempt to build a predictive model by integrating growth data, molecular expression data and FB_1

production data together, comparable studies are required on stored maize grain to establish whether similar effects occur as seen on ripening maize cobs.

5.4.2 Effects of biocontrol agents (BCAs) on *FUM1* expression and FB_1 production by *Fusarium verticillioides* FV1 on maize cobs of different ripening stages

From the maize cob experiments, three quantifiable parameters were assessed; colony development, *FUM1* gene expression and FB_1 production by the pathogenic FV1 alone and in the presence of potential biocontrol agents.

In terms of colony development of FV1 on the maize cobs, while no significant difference was obtained at 25°C across all ripening stages, at 30°C no growth was observed at R_3 stage, minimal growth at R_4 stage, and significantly smaller colony diameters were obtained at R_5 when compared to 25°C. This might suggest that 30°C was not the optimum temperature for FV1 colonization of maize cobs. Additionally, although in an earlier chapter (Chapter 2) it was shown that no significant differences were obtained in colony development of FV1 between 25 and 30°C, these data however, were obtained from *in vitro* studies only. In natural field production systems, drier and warmer conditions favour the colonization by *Fusarium* spp. during the grain filling stage (R_1 silking to R_6 maturity) that leads to *Fusarium* ear rot/cob rot (Venturini *et al*., 2015; Miller, 2001). However, information on the extent of colonization in relation to a_w and temperature in each of the maize ripening stages has not been fully documented. The maize cob experiments in the present work suggested that while the a_w between ripening stages were significantly different, the colony development of control and

treatments were not. Although not significant, the colony development in the control was always more rapid in all the ripening stages. It was also observed that as the a_w increased, so too did the colonization rate in all the treatments. Temperature appeared to have a bigger effect on colonization than the actual a_w of the maize cobs in this study.

It was also noticeable that the bacterial candidate (BCA5) had no effect on growth of FV1. This might suggest that while bacterial biocontrol agents of maize pathogens are frequently applied during soil or seed treatment (Cavaglieri *et al.*, 2005; Bacon *et al.*, 2001; Bacon and Hinton, 2000) with some inhibition, application to the maize cobs did not show such effects. The BCA5 bacterium may require higher a_w levels for active colonization and this may have perhaps hindered the competitiveness *in planta*. In contrast, in the FV1+BCA1 treatment, the colony development appeared to be mainly that of the *C. rosea* 016 than of *F. verticillioides*. BCA1 seemed to exhibit good potential in overgrowing FV1 by occupying similar a_w and temperature conditions, supported by the visible hyphal growth on the maize cobs. As mentioned previously, BCA1 is a known soil-dwelling mycoparasite (Palazzini *et al.*, 2013; Rodríguez *et* al., 2011; Mejía *et al*., 2008). Therefore, it may also be effective in soil-based treatments to reduce *F. verticillioides* inoculum in the soil.

Since fungal growth can only partially reflect the full extent of FV1 colonization on maize cobs, the *FUM1* gene expression and FB_1 production were also quantified. Generally, this showed that at 25°C, *FUM1* gene was expressed in higher amounts ($\log_{10}$ 1.4 – 4.3) as compared to 30°C ($\log_{10}$ 1.3 – 3.1). This indicates some effect of temperature on *FUM1* gene expression by FV1. Overall, *FUM1* gene expression in the

controls, irrespective of ripening stages at both temperatures, was not significantly different. Thus, over the a_w range examined in ripening maize cobs, there was no effect of a_w on *FUM1* gene expression. This is indicative of the ability of *F. verticillioides* to effectively produce FB_1 over a range of ripening conditions if it became established.

Insignificant difference observed between FV1 and FV1+BCA5 might suggest that BCA5 has very little or no effects in inhibiting *FUM1* gene expression. On the other hand, FV1+BCA1 showed significantly lower *FUM1* gene expression at all temperatures and ripening stages tested. This down-regulation of the *FUM1* gene expression confirms that there was an effect of this antagonist. This was especially so at 25°C. The slightly lower inhibition at 30°C might be because BCA1 did not grow as well at this temperature as evidenced in the lower colony development discussed earlier.

The production of FB_1 by FV1 on maize cobs of different ripening stages and temperatures was somehow more variable and less clear-cut as opposed to colony development and *FUM1* gene expression discussed earlier. In controls, as the a_w decreased across the ripening stages at 25°C, FB_1 levels steadily and significantly increased. Similar ascending pattern was also observed at 30°C but without significant difference between R_4 and R_5 (no growth was detected at R_3). These findings were in fact a known characteristic in FBs production by *Fusarium* spp. at which higher FBs are produced at drier conditions on maize cobs according to world-wide surveys (Fandohan *et al.*, 2003; Shephard *et al.*, 1996). Similar findings were also reported elsewhere (Mukanga *et al*., 2010; Bigirwa *et al.*, 2007). Furthermore, the same observations were also recorded in Chapter 3 from experiments with milled-maize agar and irradiated

maize kernels. These findings thus validated the effects of a_w on FB_1 production *in planta*.

As observed with the *FUM1* gene expression data, FV1+BCA1 treatments also gave the lowest FB_1 levels at all temperatures and ripening stages. FV1+BCA1 decreased the FB_1 levels by 73.11 ± 3.87% at 25°C, and 58.43 ± 4.14% at 30°C. However, in the case of the bacterial candidate (BCA5), although there was no significant difference between the controls in colony development and *FUM1* gene expression, there was an effect on FB_1 production. In all the treatments, with the exception of 25°C+R_3 and 30°C+R_5, there were significant reductions in FB_1 when compared with the controls. In this case, the control levels were 46.07 ± 9.14% at 25°C, and 22.34 ± 9.30% at 30°C.

Although a positive correlation was found between *FUM1* gene expression and FB_1 production across the temperatures and ripening stages, the correlation was not very strong (*i.e.*, Pearson's *r* nearing 1.00); with $r = 0.82 \pm 0.06$ at 25°C, and 0.80 ± 0.12 at 30°C. As discussed earlier, this might possibly be due to the effects of ecophysiological conditions on the expression of *FUM19* gene which encodes for an ABC (ATP-Binding Casette) transporter involved in the extracellular export of fumonisins (Proctor *et al*. 2003). In other words, even if the *FUM1* gene was highly expressed, but the *FUM19* gene was not, the actual levels of FB_1 might be affected. In the present work however, the *FUM19* gene was not quantified. Nevertheless, the data obtained in the present work are sufficient to highlight the inhibitory effects of both BCAs under the tested ecophysiological conditions.

5.5 CONCLUSIONS

Overall, the effect of colonization of maize kernels by *F. verticillioides* FV1 at 0.95 and 0.98 a_w was successfully demonstrated. It was found that by day ten, both *FUM1* gene expression and the corresponding FB_1 production were highest. Since both *FUM1* gene expression and FB_1 production fluctuate during incubation periods, selecting the suitable time for toxin quantification would certainly lower the risk of toxin underestimation. In the present study, no significant difference was obtained between days 10 and 14. Therefore, selecting the earlier incubation interval would give useful results in a shorter experimental period. This study also showed that temporal q-PCR for the *FUM1* gene expression is influenced by both a_w and temperature interactions in irradiated maize kernels, and that *FUM1* gene expression showed positive correlation with FB_1 production.

The effects of BCAs artificially inoculated onto maize cobs to lower FB_1 production by FV1 at different ripening stages and temperatures have also been successfully demonstrated. The fungal biocontrol agent *C. rosea* 016 (BCA1) significantly inhibited FB_1 levels on maize cobs by more than at 70% at 25°C, and almost 60% at 30°C irrespective of maize ripening stages. In contrast, the bacterial biocontrol agent (BCA5) lowered the FB_1 levels by almost 50% at 25°C, and more than 20% at 30°C. However, BCA5 did not appear to effectively colonize the maize cobs, and the effect on *FUM1* gene expression did not indicate effective control of FB_1. Thus, the mechanism of action of the fungal and bacterial antagonists needs more investigation.

CHAPTER 6

Overall discussion, final conclusions and future recommendations

6.1 OVERALL DISCUSSION

The infection of economically important crops such as maize by mycotoxigenic fungal pathogens, especially by members of genera *Aspergillus* and *Fusarium*, and the subsequent mycotoxin (*e.g.*, aflatoxins, fumonisins, trichothecenes) contamination are among the major problems faced by the agricultural and commercial sectors world-wide which has impacted on quality and yields (Desjardins, 2006). The clinical and health implications of such fungal toxins toward humans and animals have been widely investigated (Gelderblom *et al.*, 1988; IARC, 1993; Marasas, 2001). In the case of maize, limited host resistance to mycotoxigenic fungal pathogens, increased resistance to fungicides by fungal pathogens, and the legislative limits on chemical residue have increased application of alternative strategies for control (Whipps, 2001). Also, traditional chemical control methods of maize diseases, pests and weeds can be very expensive, relatively ineffective, and have a negative impact on human health. One of the most suitable alternatives is the use of beneficial microorganisms or biological control agents (biocontrol), which may also in some crops promote plant growth in addition to providing disease control (Zahir *et al.*, 2004). The use of biocontrol approaches has also been considered a more natural and environmentally acceptable alternative (Whipps, 2001), and as part of integrated pest management (IPM), has been suggested as the most sustainable long-term solution. Indeed, Glare *et al*. (2012) asked a pertinent question as to whether biocontrol has come of age because of the removal of

almost 50% of the agrochemicals by the EU thus providing significant drivers for the practical use of IPM or biocontrol as a major method of controlling pests and diseases.

The focus of this project was to try and identify potential biocontrol agents (bacteria or fungi) which may be able to control growth and more importantly fumonisin production by strains of *F. verticillioides* in maize-based systems and in maize cobs of different ripening stages. Thus, the present project was carried out in a series of Phases to address the overall objectives.

In Phase 1, the mycobiota diversity was analyzed for small number of maize samples from different regions (Malaysia, Mexico, France). Although the initial aim and methodology were not directed towards a comprehensive survey, the findings nevertheless reflected the structure of the mycobiota commonly isolated from maize samples from around the world (Gonzaslez *et al*., 2003; Kpodo *et al*., 2000; Marín *et al.*, 1998) with complimentary information obtained by using selective growth media. However, molecular diversity was not examined as the main driver was to obtain strains of *F. verticillioides* which produced fumonisins and to obtain indigenous microorganisms from maize kernels which could be effectively screened for antagonism against this pathogen. The diversity obtained was on harvested maize kernels only and does not reflect the temporal changes which may occur during ripening *in planta* (Fisher *et al.*, 1992). A strain of *F. verticillioides* was successfully isolated from Malaysian maize kernels and molecularly identified. This allowed some ecophysiological studies on a strain from this region to be performed in detail for the first time. This strain was coded as FV1. By using a fumonisin-inducing growth medium (FIM), it was possible to

compare FBs production of FV1 at different temperatures and a_w levels with two reference strains. This medium provided all the necessary carbon and nitrogen components which are essential in FBs production (López-Errasquín *et al.*, 2007). This showed that FV1 produced high levels of FB_1 over a range of temperatures × a_w levels. Indeed, the FB_1 production by FV1 was higher than that of the two reference strains, and thus FV1 was used in all subsequent studies.

In Phase 2, the potential antagonistic microorganisms were screened against FV1 both on maize-based medium and on stored maize kernels. One fundamental aspect in determining the potential of a biocontrol agent is to identify its antagonistic effects against target pathogens, and establish the main mechanisms involved to address the most appropriate biocontrol management strategies. As the importance of the application of indigenous microbial inoculants as biocontrol agents is being increasingly recognized (Köhl *et al.*, 2011; Xu *et al.*, 2011), several indigenous candidates have been isolated from the Malaysian maize kernels. Of these, the motile Gram-negative BCA5 gave the highest inhibition in growth rates and colony development of the pathogen, exceeding that of the three reference biocontrol candidates. However, since the growth and colonization rates are not a good indicator of the capacity for controlling mycotoxin production, it was decided to test the efficacy of biocontrol candidates in controlling FB_1 production. In these studies, a maize-based medium was used to try and simulate nutritional conditions close to the natural substrate. The findings from maize-based medium and maize kernel experiments provided a clearer picture on the efficacy of the tested BCAs and also the effects of a_w and temperature in controlling FB_1 production. Here, one fungal candidate (BCA1) and one bacterial candidate (BCA5) exhibited good

FB_1 inhibition in both maize-based medium and maize kernel experiments. Interestingly however, across the inoculum ratios (75:25 – 50:50 – 25:75) there were no significant differences in control of FB_1 levels, irrespective of a_w level tested. This suggests that in this case, inoculum of the biocontrol agent relative to the pathogen was not a very important factor in controlling FB_1 production. This is important as the less inoculum required the more economically viable a formulated product will be. This contrasts with the results obtained by Mohale *et al*. (2013), who found that at least 50:50 ratio of atoxigenic:toxigenic *A. flavus* was required for significant control of aflatoxin contamination. This would have an impact on production and formulation costs for downstream processing.

The next question was whether or not the FB_1 inhibition exhibited by both BCAs (BCA1 and BCA5) was indeed due to nutrient competition with the pathogen. Therefore, in Phase 3, the relative C-source utilization patterns of both BCAs with that of the pathogen were compared through the Niche Overlap Index (NOI) approach and Temporal Carbon Utilization Sequence (TCUS) studies. The C-sources used were the principal chemical constituents of maize kernels (Giorni *et al.*, 2009). Here, the fungal BCA1 (*C. rosea* 016) occupied similar ecological niches as the pathogen at certain ecophysiological conditions which supports the potential exclusion of the pathogen. Furthermore, BCA1 is naturally a soil-dwelling mycoparasite. Hence, there might be differences in the availability and type of CSs in the soil as compared to the maize-derived CSs used in the present work. Longer experimental time-frames might also be needed to draw up a better and more practical approach to be applied *in situ*. The failure of the bacterial BCA5 (motile Gram-negative rod) to give any measurable

growth within the experimental timeframe might be explained by the possible toxicity of PEG 600 (Kinnunen and Koskela, 1991; Cox, 1966). Of course this was an *in vitro* assay and not directly related to colonization of mixed nutrients in maize cobs, as was clearly shown later that BCA5 was effective at controlling FB_1 production on maize cobs of different ripening stages.

The colonization of *F. verticillioides* in maize occurs during the grain filling stage (R_1 silking to R_6 maturity; Venturini *et al*., 2015; Miller, 2001). Therefore, the key objectives in Phase 4 were to evaluate the potential for control of FV1 and FB_1 contamination in maize cobs of different ripening stages (R_3, R_4, R_5) which also represented different natural a_w levels and nutritional compositions. This study combined both a molecular approach (q-PCR) by quantifying a key gene in the *FUM* gene cluster involved in fumonisin production as well as effects on FB_1 control by analytical quantification (HPLC-FLD). The fungal BCA1 exhibited higher inhibition of *FUM1* expression and FB_1 production when compared with the bacterial BCA5 at all ecophysiological conditions tested. Additional studies were carried out in this phase to examine biocontrol efficacy against FV1 at 35°C in simulated climate change conditions (1,000 ppm CO_2) as opposed to 400 ppm in normal earth's atmosphere (Tans and Keeling, 2014). However, no growth of FV1 was observed in all the treatments. Moreover, 35°C has not been reported to be within the optimum range that permits *F. verticillioides* growth on maize cobs in the field. In fact, the 4–37°C temperature range reported for germination and growth of this pathogen was from *in vitro* experiments as reviewed by Marín *et al.* (2004). As observed in the experiment, 35°C favoured the growth of another fast-growing maize pathogen, *A. flavus*

(Appendix L) in all the treatments. Thus, it was not possible to ascertain the potential effects of the biocontrol agents on FB_1 production with or without climate change conditions at 35°C. In the field, the flux in temperatures significantly affects the type of fungal contaminants and mycotoxins found. Climate change is expected to have profound effects on agriculture globally (Medina *et al.*, 2014). In the case of *A. flavus*, a wide temperature range for growth 19–35°C (Northolt and van Egmond, 1981) and even wider at 12–48°C (Klich *et al.*, 1994) enable it to colonize and contaminate crops more rapidly. Abdel-Hadi *et al*. (2012) demonstrated that a strain of *A. flavus* grew optimally at 30–35°C when the a_w was 0.99, and shifted to 37°C when the a_w was lowered to 0.90. The fact that it grew in the presence of 1,000 ppm CO_2 (almost three times the normal concentration) was also corroborated by Medina *et al*. (2015) who found that climate change conditions (350–1,000 ppm CO_2; 34–37°C) have relatively little effects on the growth of *A. flavus* but significantly stimulated aflatoxin B_1 production. However, there is practically no knowledge on how climate change conditions may impact on the performance of biocontrol agents against fungal pathogens although recently, Borisade and Magan (2015) showed that the use of entomogenous fungi for the control of pests were less effective under climate change conditions.

6.2 FINAL CONCLUSIONS

On the whole, the present work has met all its objectives. The effects of ecophysiological factors on *F. verticillioides* FV1 infection on maize (growth, gene expression, toxin production) have been demonstrated on maize agar (*in vitro*), maize kernels (*in vivo*), and maize cobs (*in planta*). The efficacy of a biocontrol agent candidate (BCA1; *Clonostachys rosea* 016) has also been demonstrated in which it showed high inhibitory effects on FB_1 production *in vitro*, *in vivo* and *in planta* by *F. verticillioides* FV1. The major findings/conclusions according to Chapters are listed below.

CHAPTER 2: Biodiversity of mycobiota on maize

a) Water content and water activity of maize kernels significantly affected the mycobiota diversity and populations.

b) The dominant fungi (*Aspergillus* spp., *Fusarium* spp., *Penicillium* spp.) isolated were those normally found on cereals such as maize.

c) Molecular means were able to confirm the identity of a *Fusarium* strain isolated from Malaysian maize as *F. verticillioides* (FV1).

d) Water activity and temperature significantly affected the growth (optimum at 0.98 a_w, 25-30°C) and FB_1 production (optimum at 0.98 a_w, 25°C) by *F. verticillioides* FV1 on maize-based medium.

CHAPTER 3: Screening of antagonists for efficacy against *F. verticillioides* FV1 and FB_1 control

a) Growth and colonization rates are not a good indicator of the capacity for controlling mycotoxin production.

b) Different pathogen:antagonist inoculum ratios have inhibiting and stimulating effects on FB_1 production on maize-based medium and on maize kernels.

c) BCA1 (*Clonostachys rosea* 016) completely inhibited FB_1 production at 25:75 pathogen:antagonist inoculum ratio at 0.98 a_w on maize-based medium and on maize kernels.

d) FB_1 production was higher at 0.95 a_w when compared to 0.98 on maize-based medium and on maize kernels.

CHAPTER 4: C-source utilization patterns by NOI and TCUS

a) Water activity and temperature had impacts on C-source utilization patterns by pathogen and antagonist.

b) The TCUS results by Bioscreen complimented the traditional NOI method.

c) FV1 and BCA1 occupied similar niches at 0.95 a_w+30°C and 0.98 a_w+25°C.

d) BCA5 was unable to grow in PEG 600 perhaps because of its toxicity to the bacterial cells.

CHAPTER 5: Control of growth, *FUM1* expression and FB_1 production in maize cobs by BCAs

a) In temporal studies, both *FUM1* gene expression and FB_1 production were highest by day 10 at both 0.95 and 0.98 a_w on maize kernels.

b) Water activity and temperature had significant impacts on growth, *FUM1* gene expression and FB_1 production by *F. verticillioides* FV1 on maize cobs of different ripening stages.

c) BCA1 significantly reduced FB_1 levels on maize cobs by more than at 70% at 25°C, and almost 60% at 30°C irrespective of maize ripening stage.

d) BCA5 was able to inhibit FB_1 production on maize cobs although growth and *FUM1* gene expression were not affected.

6.3 FUTURE RECOMMENDATIONS

(a) Potential for development of effective formulations for use either as: (1) a spray inoculum, (2) a treatment for crop residue, or (3) a seed coating to control the endophytic component of the pathogen's life cycle.

(b) Application of different formulations to maize cobs during silking to examine whether natural colonization would be able to protect against infection by *F. verticillioides.*

(c) Colonization of silks and ability to prevent *F. verticillioides* colonization by using molecular tracking of pathogen growth or perhaps using a GFP-marked strain for this purpose.

(d) Further identification of the Gram-negative rod bacterium BCA5 and possible enhancement in its inhibitory properties towards the pathogen, and possible development of formulations for application and testing at different stages of life cycle of the pathogen.

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PUBLICATION AND TRAINING

1. **Samsudin, N. I. P.** and Magan, N. (2015). Efficacy of potential biocontrol agents for control of *Fusarium verticillioides* and fumonisin B_1 under different environmental conditions. Accepted: May 21st, 2015. Published Online: September 30th, 2015. ***World Mycotoxin Journal*.**

2. **Samsudin, N. I. P.**, Rodríguez, A. and Magan, N. (2015). Ecophysiological determinants of growth, FB_1 biosynthesis and *FUM1* gene expression by a Malaysian strain of *Fusarium verticillioides* on maize agar and maize kernels. ***Submitted Sept 2015***.

3. **Samsudin, N. I. P.**, Medina, A. and Magan, N. (2015). Temperature and water availability affect carbon utilisation patterns and niche overlap of biocontrol *Clonostachys rosea* 016 and toxigenic *Fusarium verticillioides*.
   Submitted Sept 2015.

4. **Samsudin, N. I. P.**, Medina, A., Rodríguez, A. and Magan, N. (2015). Effects of biocontrol agents in controlling fumonisin B_1 production by *Fusarium verticillioides* FV1 in maize cobs of different ripening stages by monitoring both *FUM1* gene expression and phenotypic toxin production. ***In progress.***

4. Abstract submitted, and poster presented at the *British Mycological Society (BMS) Annual Scientific Meeting, 10th-13th September 2013, Cardiff,* ***WALES***.

CRANFIELD HEALTH

Cranfield UNIVERSITY

Screening of indigenous microflora of maize for biocontrol agents against *Fusarium verticillioides* growth *in vitro*

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INTRODUCTION

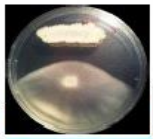
- *Fusarium verticillioides* in an important pathogen of maize because it contaminates the cobs with the fumonisins
- There is strict legislation on the maximum fumonisin levels which are acceptable in maize
- They are hepatotoxic, nephrotoxic and carcinogenic in nature
- There is interst in developing biological control apporaches for the control of *F.verticillioides* and reduction of fumonisins in maize
- The present work was undertaken to screen indigenous microflora isolated from maize which could control growth and fumonisin production by *F.verticillioides* in vitro.

MATERIALS AND METHODS

Fungal isolation (direct plating) of *F. verticillioides*

CODE	ANTAGONIST
A1	*Bacterium*
A2	*Yeast*
A3	*Streptomyces* sp. AS1
A4	*Yeast*
A5	*Yeast*
A6	*Gram-negative bacterium*

Isolation of antagonists

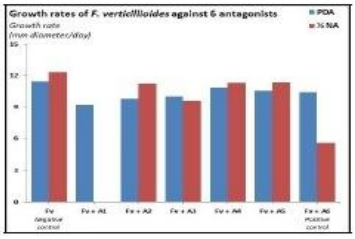


Co-inoculation of *F. verticillioides* with antagonists

Hyphal extension measurement (diameter; mm) at day 1, 3, 5, 7

- Growth rate *(mm/d)*
- Hyphal area *(cm^2)*
- Inhibition *(%)*

RESULTS



CULTURE	PDA GROWTH RATE	PDA INHIBITION (%)	½NA GROWTH RATE	½NA INHIBITION (%)	PDA HYPHAL AREA (cm^2)	½NA HYPHAL AREA (cm^2)
Fv	11.44	-	12.31	-	***45.38***	***56.77***
Fv + BCA1	9.2	***19.6***	-	-	***30.40***	-
Fv + BCA2	9.8	***14.3***	11.19	***9.1***	***36.33***	***46.10***
Fv + BCA3	9.98	***12.8***	9.57	***22.3***	***35.06***	***29.05***
Fv + BCA4	10.85	***5.2***	11.32	***8.0***	***35.91***	***37.41***
Fv + BCA5	10.52	***8.0***	11.36	***7.7***	***34.85***	***38.72***
Fv + BCA6	10.38	***9.3***	5.51	***55.2***	***35.06***	***9.63***

DISCUSSION

- Yeasts (BCAs 2,4,5) exhibited low growth rate inhibition ranging from 5.2 to 14.3% on both PDA and NA.
- Gram-negative bacterium (BCA6) inhibited growth rate of *F. verticillioides* by 55.2% on NA, exceeding that of the positive control (BCA3; *Streptomyces* sp. AS1., 22.3%).
- In terms of total hyphal area, BCA6 inhibited the total hyphal growth of *F. verticillioides* to 9.63 cm^2 as compared to that of positive control (29.05 cm^2).
- Different values of inhibition between yeasts and bacteria are due to different media favouring different growth of microorganisms, with bacteria growing better on NA, and yeasts and moulds on PDA.
- Overall, BCA5 inhibited the growth of *F. verticillioides* the most as measured by growth rate (mm/d), inhibition (%), and hyphal area (cm^2), and was selected as potential biocontrol agent for examining effects on fumonisin production.

FUTURE WORK

- Effects of BCA5 antagonism under different environmental factors (a_w, temperature) and on fumonisin biosynthesis by *F.verticillioides*
- Spore-ratio inoculation (100:0, 75:25, 50:50, 25:75, 0:100) on milled maize agar-MMA (*in vitro*), and on maize cobs (*in vivo*)

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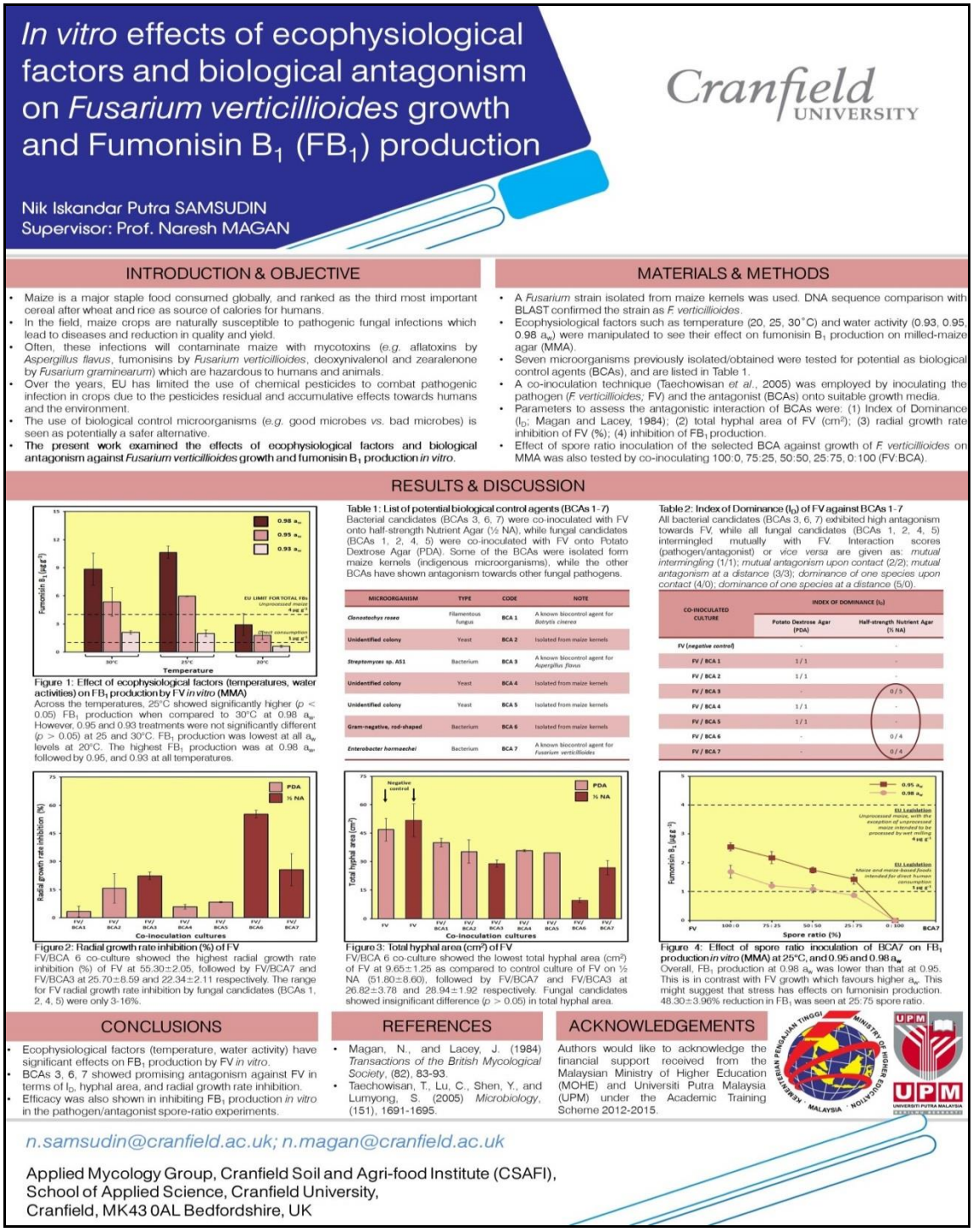

In vitro effects of ecophysiological factors and biological antagonism on *Fusarium verticillioides* growth and Fumonisin B_1 (FB_1) production

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INTRODUCTION & OBJECTIVE

- Maize is a major staple food consumed globally, and ranked as the third most important cereal after wheat and rice as source of calories for humans.
- In the field, maize crops are naturally susceptible to pathogenic fungal infections which lead to diseases and reduction in quality and yield.
- Often, these infections will contaminate maize with mycotoxins (*e.g.* aflatoxins by *Aspergillus flavus*, fumonisins by *Fusarium verticillioides*, deoxynivalenol and zearalenone by *Fusarium graminearum*) which are hazardous to humans and animals.
- Over the years, EU has limited the use of chemical pesticides to combat pathogenic infection in crops due to the pesticides residual and accumulative effects towards humans and the environment.
- The use of biological control microorganisms (*e.g.* good microbes *vs.* bad microbes) is seen as potentially a safer alternative.
- **The present work examined the effects of ecophysiological factors and biological antagonism against *Fusarium verticillioides* growth and fumonisin B_1 production *in vitro*.**

MATERIALS & METHODS

- A *Fusarium* strain isolated from maize kernels was used. DNA sequence comparison with BLAST confirmed the strain as *F. verticillioides*.
- Ecophysiological factors such as temperature (20, 25, 30˚C) and water activity (0.93, 0.95, 0.98 a_w) were manipulated to see their effect on fumonisin B_1 production on milled-maize agar (MMA).
- Seven microorganisms previously isolated/obtained were tested for potential as biological control agents (BCAs), and are listed in Table 1.
- A co-inoculation technique (Taechowisan *et al.*, 2005) was employed by inoculating the pathogen (*F. verticillioides*; FV) and the antagonist (BCAs) onto suitable growth media.
- Parameters to assess the antagonistic interaction of BCAs were: (1) Index of Dominance (I_D; Magan and Lacey, 1984); (2) total hyphal area of FV (cm^2); (3) radial growth rate inhibition of FV (%); (4) inhibition of FB_1 production.
- Effect of spore ratio inoculation of the selected BCA against growth of *F. verticillioides* on MMA was also tested by co-inoculating 100:0, 75:25, 50:50, 25:75, 0:100 (FV:BCA).

RESULTS & DISCUSSION

Figure 1: Effect of ecophysiological factors (temperatures, water activities) on FB_1 production by FV *in vitro* (MMA)
Across the temperatures, 25°C showed significantly higher ($p < 0.05$) FB_1 production when compared to 30°C at 0.98 a_w. However, 0.95 and 0.93 treatments were not significantly different ($p > 0.05$) at 25 and 30°C. FB_1 production was lowest at all a_w levels at 20°C. The highest FB_1 production was at 0.98 a_w, followed by 0.95, and 0.93 at all temperatures.

Table 1: List of potential biological control agents (BCAs 1-7)
Bacterial candidates (BCAs 3, 6, 7) were co-inoculated with FV onto half-strength Nutrient Agar (½ NA), while fungal candidates (BCAs 1, 2, 4, 5) were co-inoculated with FV onto Potato Dextrose Agar (PDA). Some of the BCAs were isolated form maize kernels (indigenous microorganisms), while the other BCAs have shown antagonism towards other fungal pathogens.

MICROORGANISM	TYPE	CODE	NOTE
Clonostachys rosea	Filamentous fungus	BCA 1	A known biocontrol agent for *Botrytis cinerea*
Unidentified colony	Yeast	BCA 2	Isolated from maize kernels
Streptomyces sp. A51	Bacterium	BCA 3	A known biocontrol agent for *Aspergillus flavus*
Unidentified colony	Yeast	BCA 4	Isolated from maize kernels
Unidentified colony	Yeast	BCA 5	Isolated from maize kernels
Gram-negative, rod-shaped	Bacterium	BCA 6	Isolated from maize kernels
Enterobacter hormaechei	Bacterium	BCA 7	A known biocontrol agent for *Fusarium verticillioides*

Table 2: Index of Dominance (I_D) of FV against BCAs 1-7
All bacterial candidates (BCAs 3, 6, 7) exhibited high antagonism towards FV, while all fungal candidates (BCAs 1, 2, 4, 5) intermingled mutually with FV. Interaction scores (pathogen/antagonist) or *vice versa* are given as: *mutual intermingling* (1/1); *mutual antagonism upon contact* (2/2); *mutual antagonism at a distance* (3/3); *dominance of one species upon contact* (4/0); *dominance of one species at a distance* (5/0).

CO-INOCULATED CULTURE	INDEX OF DOMINANCE (I_D)	
	Potato Dextrose Agar (PDA)	Half-strength Nutrient Agar (½ NA)
FV (*negative control*)	-	-
FV / BCA 1	1 / 1	-
FV / BCA 2	1 / 1	-
FV / BCA 3	-	0 / 5
FV / BCA 4	1 / 1	-
FV / BCA 5	1 / 1	-
FV / BCA 6	-	0 / 4
FV / BCA 7	-	0 / 4

Figure 2: Radial growth rate inhibition (%) of FV
FV/BCA 6 co-culture showed the highest radial growth rate inhibition (%) of FV at 55.30±2.05, followed by FV/BCA7 and FV/BCA3 at 25.70±8.59 and 22.34±2.11 respectively. The range for FV radial growth rate inhibition by fungal candidates (BCAs 1, 2, 4, 5) were only 3-16%.

Figure 3: Total hyphal area (cm^2) of FV
FV/BCA 6 co-culture showed the lowest total hyphal area (cm^2) of FV at 9.65±1.25 as compared to control culture of FV on ½ NA (51.80±8.60), followed by FV/BCA7 and FV/BCA3 at 26.82±3.78 and 28.94±1.92 respectively. Fungal candidates showed insignificant difference ($p > 0.05$) in total hyphal area.

Figure 4: Effect of spore ratio inoculation of BCA7 on FB_1 production *in vitro* (MMA) at 25°C, and 0.95 and 0.98 a_w
Overall, FB_1 production at 0.98 a_w was lower than that at 0.95. This is in contrast with FV growth which favours higher a_w. This might suggest that stress has effects on fumonisin production. 48.30±3.96% reduction in FB_1 was seen at 25:75 spore ratio.

CONCLUSIONS

- Ecophysiological factors (temperature, water activity) have significant effects on FB_1 production by FV *in vitro*.
- BCAs 3, 6, 7 showed promising antagonism against FV in terms of I_D, hyphal area, and radial growth rate inhibition.
- Efficacy was also shown in inhibiting FB_1 production *in vitro* in the pathogen/antagonist spore-ratio experiments.

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6. Abstract submitted, and poster presented at the *10th International Mycological Congress (IMC), 3rd – 8th August 2014, Bangkok,* ***THAILAND***.

Reduction in fumonisin B_1 (FB_1) biosynthesis by *Fusarium verticillioides* co-cultivated with different inoculum ratios of biological control agents *in vitro*

NIK ISKANDAR PUTRA SAMSUDIN
Supervisor: Prof. Naresh Magan

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SPONSORS:


FIGURE 1 : Healthy maize


FIGURE 2 : Infected maize

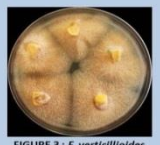
FIGURE 3 : *F. verticillioides* isolated from maize kernels

INTRODUCTION & OBJECTIVES

- In the field, infection of *F. verticillioides* in maize occurs horizontally and vertically.
- Horizontally, *F. verticillioides* colonize the rhizosphere (root systems) and spermosphere (seedling surface) and infect the maize seedlings (seedborne infection), resulting in an asymptomatic (symptomless) and systemic infection throughout the lifecycle of the maize crops.
- Factors controlling the interchanging between symptomatic and asymptomatic infection are not yet fully understood at present.
- Vertically, *F. verticillioides* is dispersed by wind (airborne infection), or by means of kernel wounds inflicted by birds or insects, resulting in rapid infection of maize kernels and cobs, which contributes to loss in yield.
- Surface treatments of seedlings is a method to reduce the number of disease-causing fungi and bacteria found on the seedlings, either physically, chemically or biologically.
- Biologically, microbial inoculum (spores or propagules) is used to parasitize the pathogens, compete for nutrients, or produce toxic compounds that inhibit the pathogens.
- However, microbial inoculum which is applied to the rhizosphere can either inhibit or stimulate the growth of pathogens (stress defence mechanism).
- **Hence, in the present work, the optimum inoculum ratio of pathogen:antagonist to inhibit fumonisin B_1 (FB_1) biosynthesis by *F. verticillioides in vitro* was explored.**

MATERIALS & METHODS

- A strain of molecularly-identified *Fusarium verticillioides* (FV) isolated from maize kernels was used.
- Four microorganisms selected from preliminary screening for antagonism were tested for potential against FB_1 reduction by FV (Table 1).
- Five inoculum ratios (pathogen:antagonist) of 100:0, 75:25, 50:50, 25:75, 0:100 were prepared at a concentration of 10^6 spores/mL inoculum.
- FB_1 analysis was performed according to AOAC (2001) with pre-column derivatization of samples with OPA (*ortho-phthaldialdehyde*), before being analyzed by FLD-HPLC.

Milled maize agar (0.95, 0.98 a_w) → 25°C 14 days → Solvent extraction (hyphal plugs) → HPLC

TABLE 1: List of potential biocontrol agents (BCAs) used against *F. verticillioides* (FV)

MICROORGANISM	CODE	NOTE
Clonostachys rosea 016 (fungus)	BCA 1	A known biocontrol agent for *Botrytis cinerea* (infecting grape, strawberry, tomato). A saprophyte of soil and crops residue (Palazzini *et al.*, 2013).
Streptomyces sp. AS1 (bacterium)	BCA 3	A known biocontrol agent for *Aspergillus flavus*. Significantly reduced *A. flavus* growth and aflatoxin B1 level during co-cultivation (Sultan & Magan, 2011).
Gram-negative rod (bacterium)	BCA 6	Indigenous strain isolated from maize kernels (in-house isolation). Highly motile. Exhibited promising antagonism during preliminary screening.
Enterobacter hormaechei (bacterium)	BCA 7	A known biocontrol agent for *Fusarium verticillioides*. Gram-negative, rod-shaped. Can be found on human skin and vegetable/fruit surface (Pereira *et al.*, 2010).

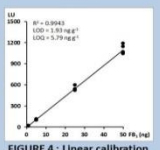

FIGURE 4 : Linear calibration curve, LOD and LOQ for FB_1

RESULTS & DISCUSSION

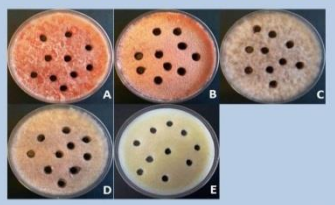

FIGURE 5 : *In vitro* interaction between FV (pathogen) and BCA (antagonist) at different inoculum ratios (FV:BCA) on MMA at 0.98 a_w
(A) 100:0; positive control, (B) 75:25, (C) 50:50, (D) 25:75, and (E) 0:100; negative control. The growth of FV visibly decreased with an increase in the antagonists inoculum ratio. Competition for nutrients or space with the antagonists might have occurred at this point. Similar trends of declining growth of FV was also noted at 0.95 a_w except that growth of FV was generally more sparse at 0.95 a_w as compared to 0.98 a_w for all BCAs, and for all inoculum ratios tested. This might suggest that FV requires higher a_w for growth (Giorni *et al.*, 2008).

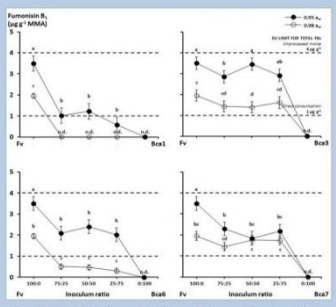

FIGURE 6 : FB_1 biosynthesis by FV co-cultivated with BCAs 1, 3, 6, 7 at different inoculum ratios.
FB_1 biosynthesis was higher at 0.95 a_w instead of 0.98 a_w (which exhibited more growth of FV) for all BCAs and inoculum ratios tested. This is due to the fact that fungal growth and FB_1 biosynthesis is not parallel. FB_1 was found to be below the EU limit for unprocessed maize but exceeded the limit for direct human consumption except for BCA1 and BCA6 which reduced FB_1 biosynthesis below the limit (1 μg g^{-1}).

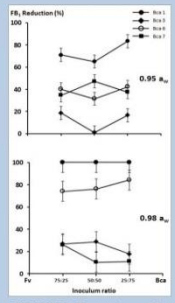

FIGURE 7 : Percentage of FB_1 reduction by different BCAs, and at different inoculum ratios
BCA1 exhibited the highest reduction of FB_1 (100% at 0.98 a_w, 73% at 0.95 a_w) followed by BCA6 (78% at 0.98 a_w, 38% at 0.95 a_w).

CONCLUSIONS

- *Fusarium verticillioides* growth and fumonisin B_1 biosynthesis were not parallel. Growth was faster at 0.98 a_w but FB_1 biosynthesis was higher at 0.95 a_w.
- Available waters (0.95, 0.98 a_w) significantly affected FB_1 biosynthesis.
- Different inoculum ratios either reduced or stimulated FB_1 biosynthesis.
- *Clonostachys rosea* 016 reduced FB_1 biosynthesis by 73% (0.95 a_w) and 100% (0.98 a_w).

FUTURE WORK

- Similar effects of available waters and different inoculum ratios are currently being tested on irradiated maize kernels (*in vivo*).
- Results from *in vitro* and *in vivo* experiments might elucidate the impacts of antagonism and ecophysiological factors on FB_1 reduction in FV.
- We will also examine the relative biocontrol efficacy in maize cobs of different ripening stages between silking and harvest.

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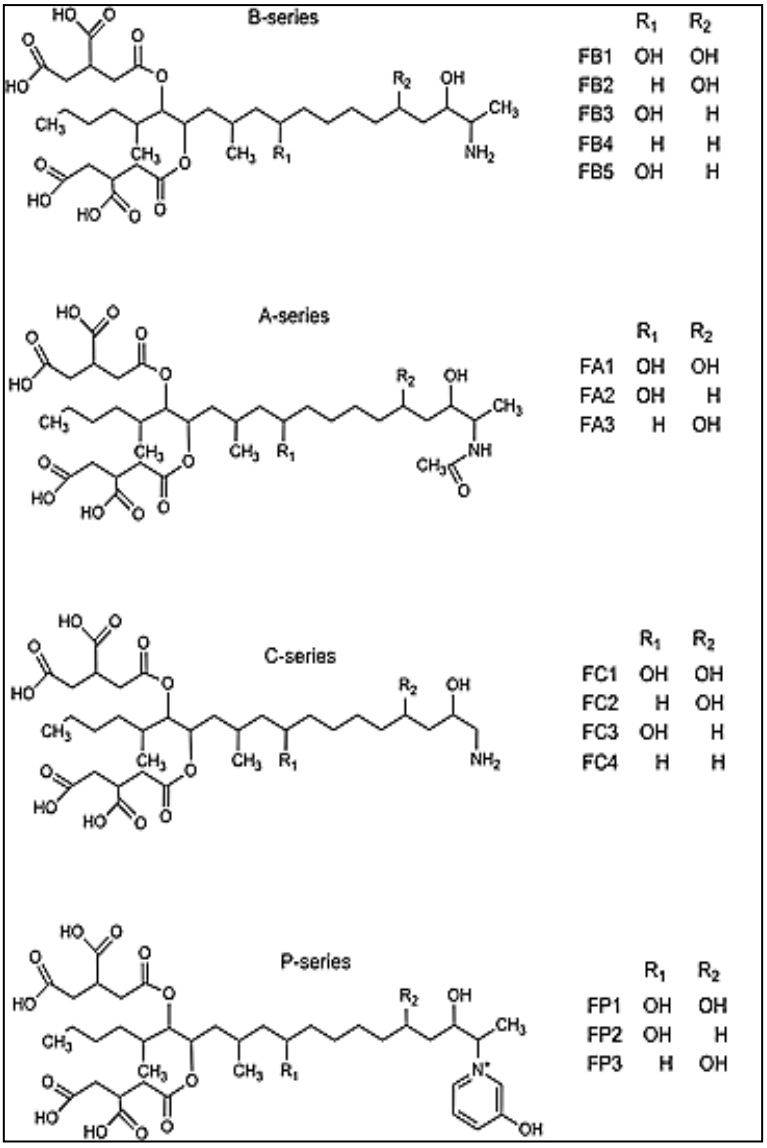
7. A workshop attended at the *ERASMUS INTENSIVE PROGRAMME (IP) on the "Methods in Food Mycology and Mycotoxicology", 8th – 22nd June 2014, Bragança, **PORTUGAL***.

8. Various short-courses on research core skills organized by the Cranfield Doctoral Training Centre (DTC) such as "*Technical Research Writing*", "*Experimental Designs*", "*Communicating Your Research*", and "*Computer Skills in Research (Microsoft Word, Microsoft Excel, and Microsoft PowerPoint)*".

9. Various taught modules/seminars attended such as "*Food Mycology*", "*Mycotoxicology*", "*Molecular Biology*", "*Chromatography*" and "*Statistics*".

APPENDIX A: Fumonisin series (Bs, As, Cs, Ps) produced by *Fusarium verticillioides* in culture media and in maize kernels (adapted from Falavigna *et al.*, 2012)



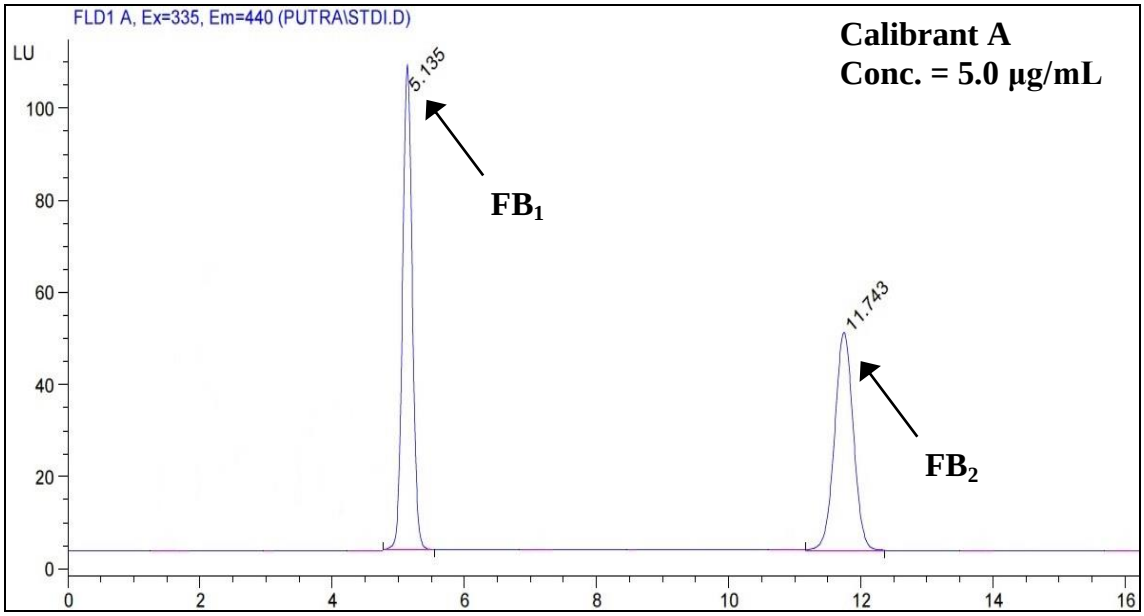
APPENDIX B: List of commonly used fungicides as provided by the Health and Safety Executive, (HSE, 2015)

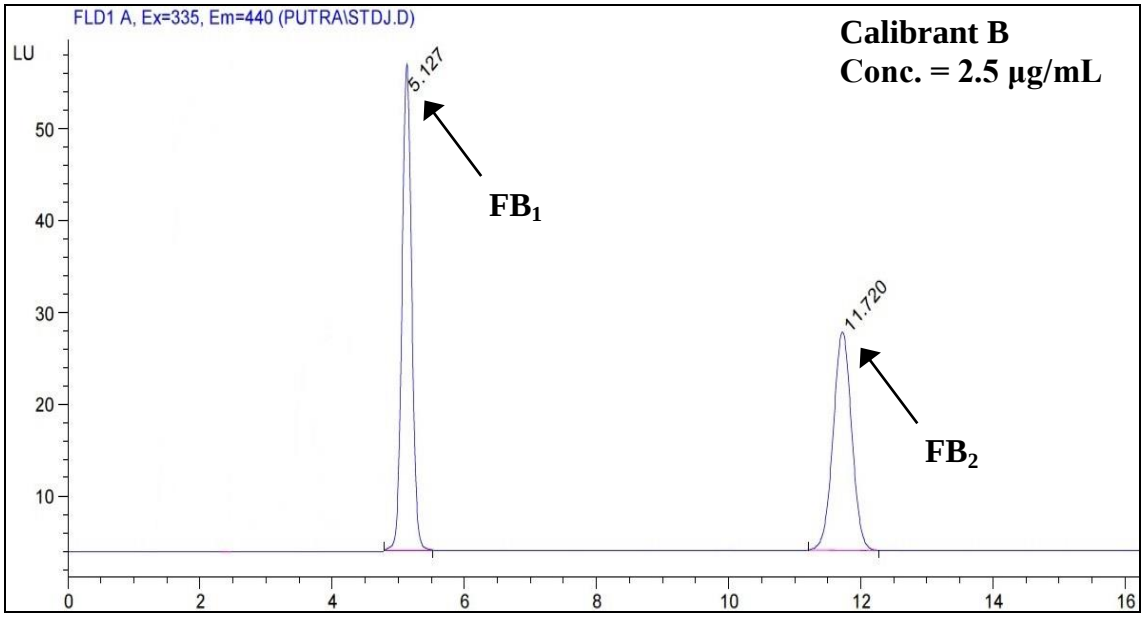
Acibenzolar-S-methyl
Benalaxyl
Biphenyl
Bitertanol
Boscalid
Bupirimate
Captan
Carbendazim
Chlorothalonil
Cyazofamid
Cyflufenamid
Cymoxanil
Cyproconazole
Cyprodinil
Dicloran
Diethofencarb
Difenoconazole
Dimethomorph
Dimoxystrobin
Diphenylamine
Dithianon
Dithiocarbamates[1]
Dodine
Epoxiconazole
Fenamidone
Fenarimol
Fenbuconazole
Fenhexamid
Fenpropimorph
Fluazinam
Fludioxonil
Fluopicolide
Fluoxastrobin
Flusilazole
Folpet
Furalaxyl
Hexachlorobenzene
Hexaconazole
Imazalil
Iprodione[2]
Iprovalicarb
Kresoxim-methyl
Mepanipyrim
Metalaxyl
Metrafenone
Myclobutanil
Oxadixyl
Penconazole
Pencycuron
Picoxystrobin
Prochloraz
Procymidone
Propamocarb
Propiconazole
Pyraclostrobin
Pyrifenox
Pyrimethanil
Quinoxyfen
Quintozene
Spiroxamine
Tebuconazole
Tebufenozide
Tecnazene
Tetraconazole
Thiabendazole
Thiophanate-methyl
Tolclofos-methyl
Tolylfluanid
Triadimefon
Triadimenol
Trifloxystrobin
Vinclozolin
Zoxamide

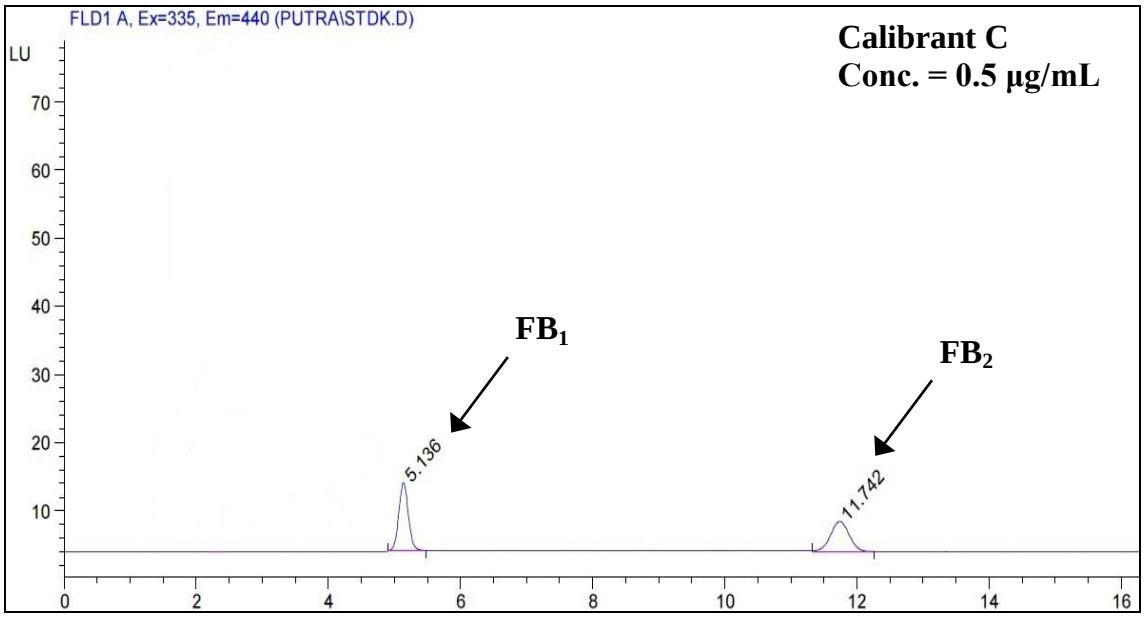
[1]Dithiocarbamates are a group of fungicides that have a similar chemical structure which include maneb, mancozeb, metiram, propineb, thiram, ziram and zineb.

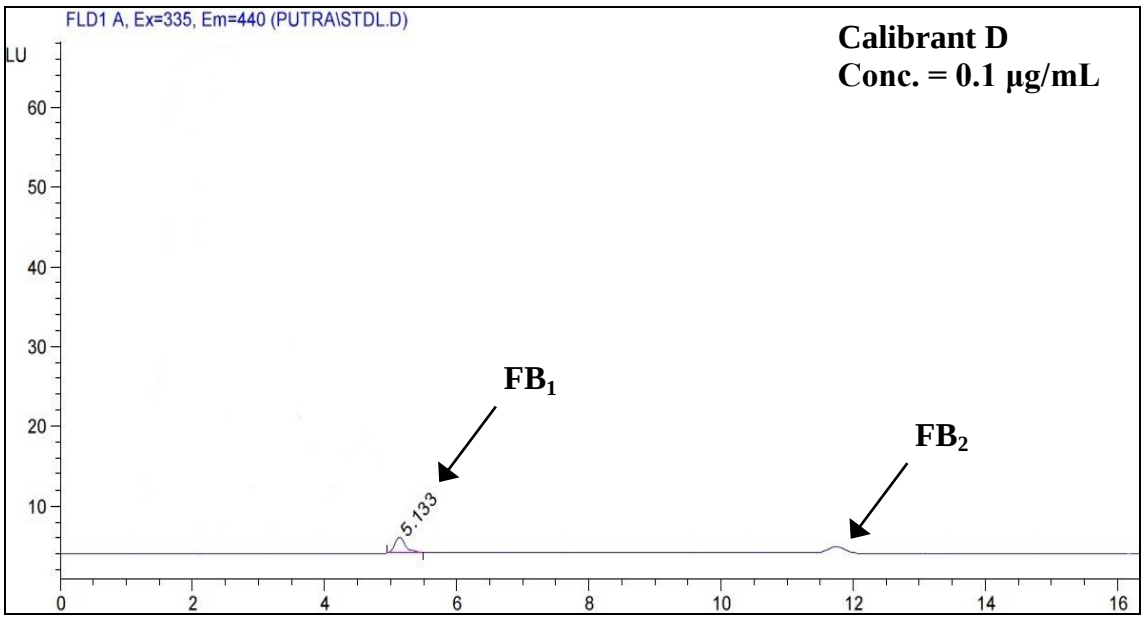
[2]Iprodione is a fungicide used to control fungi on a wide range of fruits, vegetables and cereals. It is sometimes found on UK apples. Though its levels are not illegal or a risk to health, it is not approved for use on apples in the UK. However, since it is approved for use on pears in the UK, the residues found on apples may come from cross-contamination from pears after harvest rather than from illegal use.

APPENDIX C: Chromatograms for calibrant solutions with retention times obtained through HPLC-FLD (Agilent, UK) as provided by the ChemStation software (Agilent, UK)





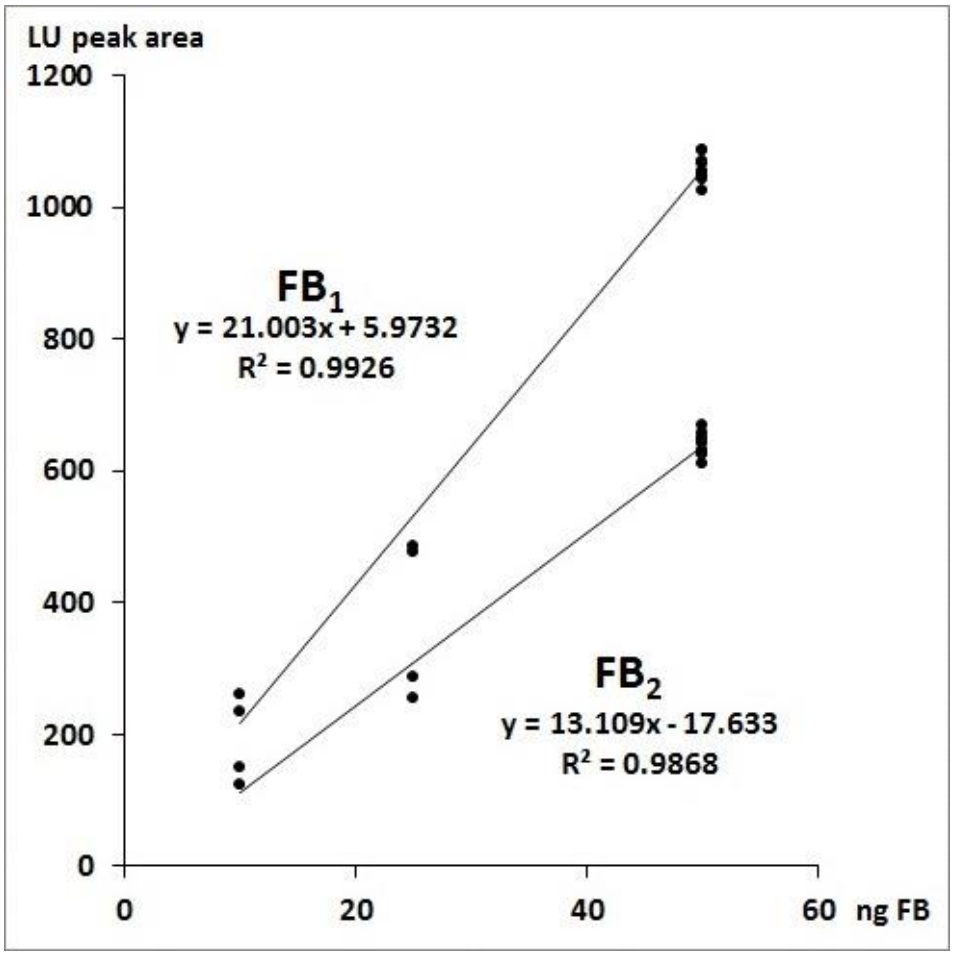




Calibrant	Conc. (µg/mL)	Injection (30 µL)*	Injected Weight	Retention Time (FB_1)	LU Area (FB_1)	Retention Time (FB_2)	LU Area (FB_2)
A	5.0	10	50 ng	5.135	**1072.0**	11.743	**930.7**
B	2.5	10	25 ng	5.127	**535.7**	11.720	**463.4**
C	0.5	10	5 ng	5.136	**101.5**	11.742	**86.3**
D	0.1	10	1 ng	5.133	**22.0**	11.735	**18.1**

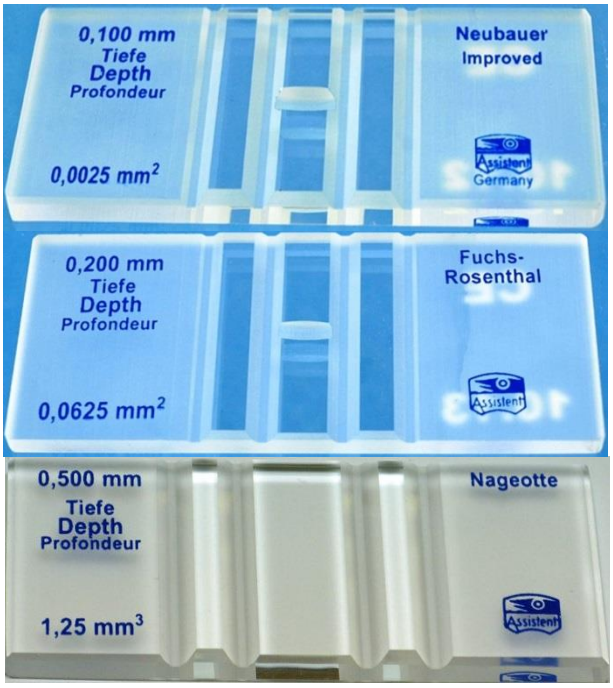
* 20 µL OPA + 10 µL Calibrant

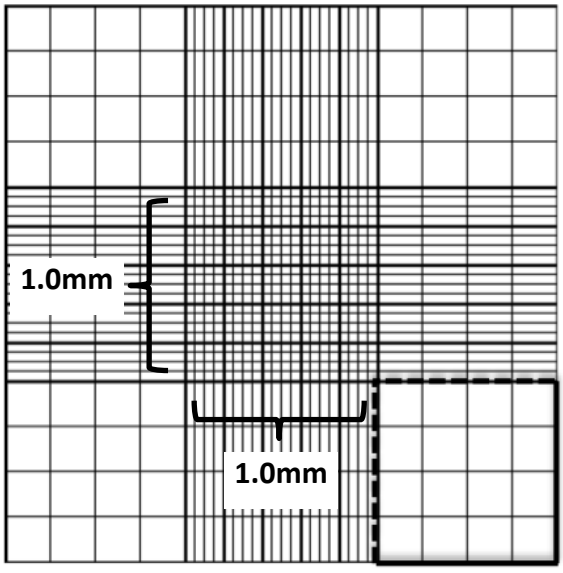
APPENDIX D: Standard curve for the determination of fumonisin B_1 and B_2



APPENDIX E: Enumeration of cells/spores by haemocytometer

The hemocytometer is a device used to count cells. It was originally designed for the counting of blood cells. The hemocytometer was invented by Louis-Charles Malassez and consists of a thick glass microscope slide with a rectangular indentation that creates a chamber. This chamber is engraved with a laser-etched grid of perpendicular lines. The device is carefully crafted so that the area bounded by the lines is known, and the depth of the chamber is also known. It is therefore possible to count the number of cells or particles in a specific volume of fluid, and thereby calculate the concentration of cells in the fluid overall. Commercially, several types of haemocytometers are available. What differs in all of them is the depth of the loading chamber which influences with the overall measurement; Neubauer (0.1 mm; 0.0025 mm^2 area), Helber (which is used in the present work, 0.2 mm; 0.0625 mm^2 area), and Nageotte (0.5 mm; 1.25 mm^2 area). Basically, the counting grid is as described below (*right*).



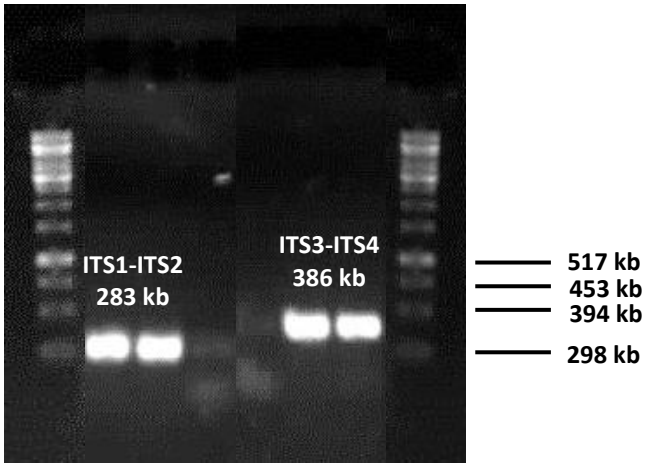


Haemocytometer has a 1.0mm x 1.0mm large square with 25 smaller squares etched in it. So, the area of that 25 smaller squares is = 0.2mm x 0.2mm = 0.04 mm^2.

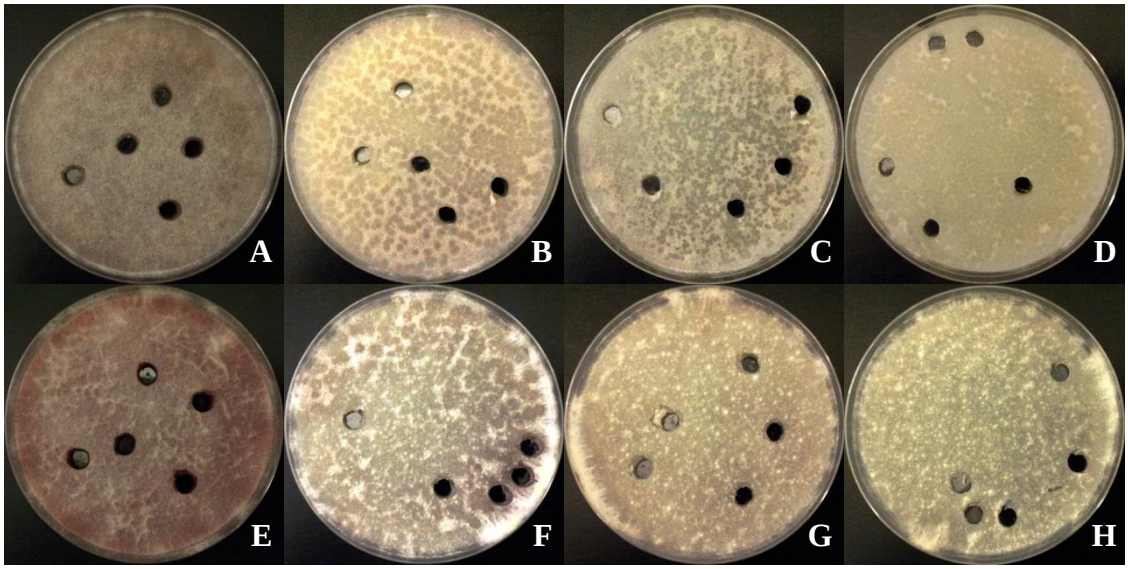
Inside that 25 smaller square (0.04 mm^2), there are 16 smallest squares with the area of 0.04 mm^2 ÷ 16 = 0.0025mm^2, which are usually used to count spores/cells.

Therefore, depending on the depth of your chosen haemocytometer, you can then determine the volume of your sample drop together with the number of spores/cells in it before adjusting in to per mL with the formula 1 µL = 1mm^3.

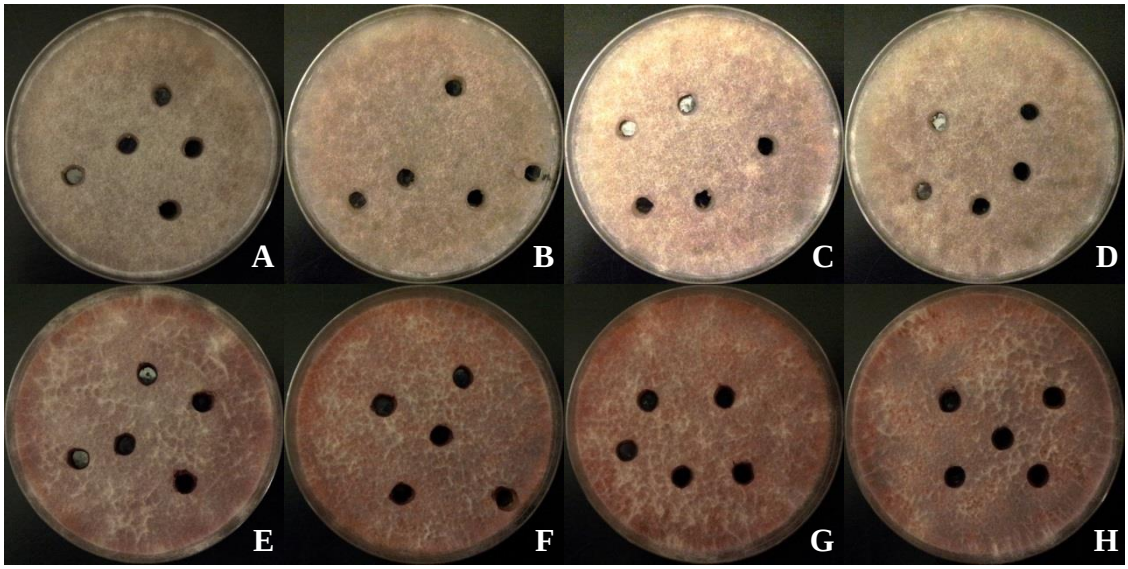
APPENDIX F: Electrophoresis gel for PCR amplification with primers ITS1-2 and ITS3-4 on DNA of *Fusarium verticillioides* FV1



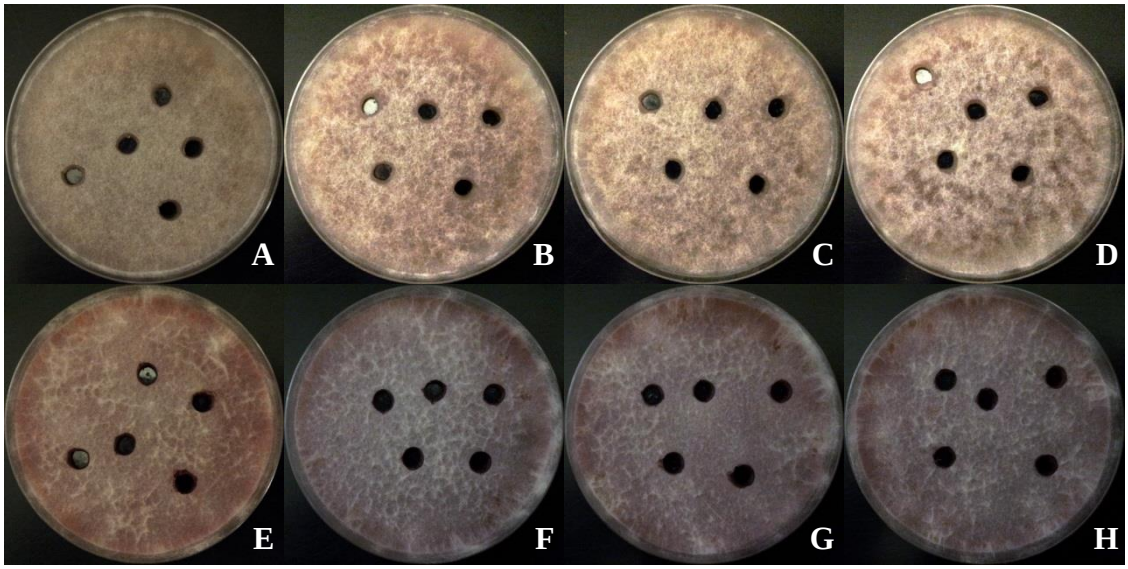
APPENDIX G: Milled-maize agar inoculated with *F. verticillioides* FV1 and BCAs at different FV:BCA inoculum ratios (*in vitro*)



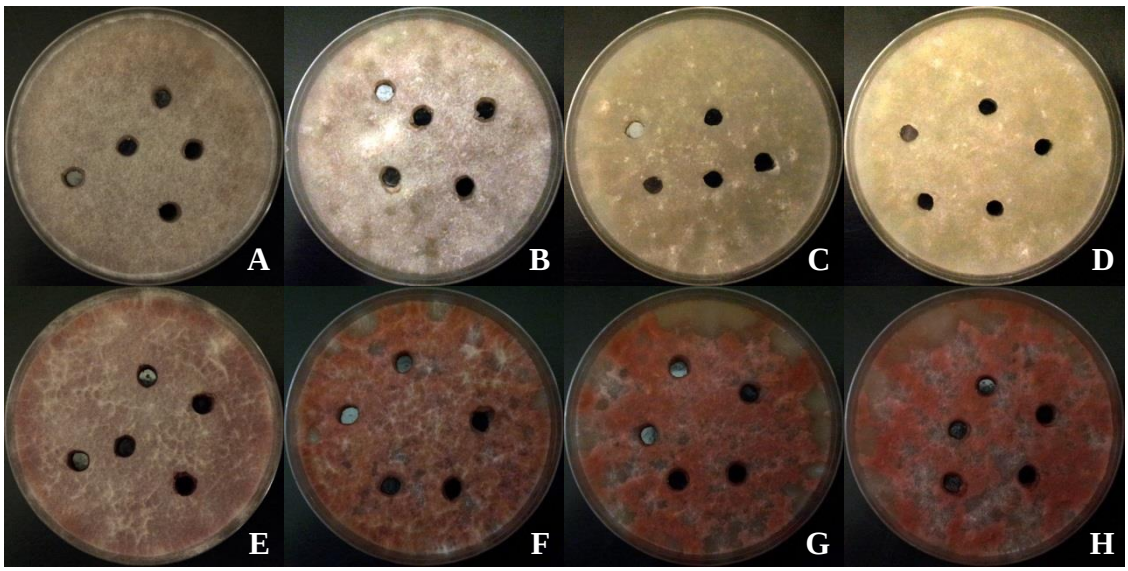
Growth of FV1 with BCA1 at different inoculum ratios on milled-maize agar (MMA) at 0.95 a_w (above) and 0.98 a_w (below). A, E (100:0, positive control); B, F (75:25); C, G (50:50) and D, H (25:75). No hyphal growth of FV1 (reddish mycelia) on plates with BCA1.



Growth of FV1 with BCA4 at different inoculum ratios on milled-maize agar (MMA) at 0.95 a_w (above) and 0.98 a_w (below). A, E (100:0, positive control); B, F (75:25); C, G (50:50) and D, H (25:75). Denser hyphal growth of FV1 is noted at 0.98 a_w as compared to 0.95 a_w.



Growth of FV1 with BCA5 at different inoculum ratios on milled-maize agar (MMA) at 0.95 a_w (above) and 0.98 a_w (below). A, E (100:0, positive control); B, F (75:25); C, G (50:50) and D, H (25:75). Denser hyphal growth of FV1 is noted at 0.98 a_w as compared to 0.95 a_w.



Growth of FV1 with BCA6 at different inoculum ratios on milled-maize agar (MMA) at 0.95 a_w (above) and 0.98 a_w (below). A, E (100:0, positive control); B, F (75:25); C, G (50:50) and D, H (25:75). Denser hyphal growth of FV1 is noted at 0.98 a_w as compared to 0.95 a_w.

APPENDIX H: Irradiated maize kernels inoculated with *F. verticillioides* FV1 and BCAs at different FV1:BCA inoculum ratios (*in vivo*)

100:0

75:25

50:50

25:75

APPENDIX I: Preparation of Polyethylene Glycol 600 (PEG 600) isopleth

Various concentrations of PEG 600 solution were prepared by adding distilled water according to the table below. Water activity of the solutions (three replicates each) at room temperature was then measured with an electronic water activity analyser (Series 3, V1.7, AquaLab, USA). Prior to this, a solution with a known water activity was prepared and measured to calibrate the analyser. During measurements of PEG 600 solutions, activated charcoal (provided with the analyser) was also run in the measuring chamber in between measurements to absorb the condensation on the mirror inside the measuring chamber. Water activity readings of PEG 600 solutions were adjusted with the calibration factors, and averaged. A graph of water activity on the *y-axis* was then plotted with the averaged readings against solute percentage on the *x-axis*.

H_2O (mL)	**PEG 600 (mL)**	**Solute % (v/v)**	**Final volume (mL)**
20	**0**	**0**	20
19	**1**	**5**	20
18	**2**	**10**	20
17	**3**	**15**	20
16	**4**	**20**	20
15	**5**	**25**	20
14	**6**	**30**	20
13	**7**	**35**	20
12	**8**	**40**	20
11	**9**	**45**	20
10	**10**	**50**	20
9	**11**	**55**	20
8	**12**	**60**	20
7	**13**	**65**	20
6	**14**	**70**	20
5	**15**	**75**	20
4	**16**	**80**	20
3	**17**	**85**	20
2	**18**	**90**	20
1	**19**	**95**	20
0	**20**	**100**	20

APPENDIX J: Type and description of 24 carbon sources used in NOI and TCUS experiments

Amino acids

	Molecular mass	Type
L-Aspartic acid	133.10 g/mol	Acidic
L-Glutamic acid	147.13 g/mol	
D-Alanine	89.09 g/mol	Aliphatic (non-polar, hydrophobic)
L-Alanine	89.09 g/mol	
L-Leucine	131.17 g/mol	
L-Phenylalanine	165.19 g/mol	Aromatic
L-Histidine	155.15 g/mol	Basic
L-Proline	115.13 g/mol	Cyclic
D-Serine	105.09 g/mol	Hydroxyl (polar, hydrophilic)
L-Serine	105.09 g/mol	
D-L-Threonine	119.12 g/mol	

Carbohydrates

	Type		Mol. mass
D-Fructose	Monosaccharide		180.16 g/mol
D-Galactose			180.16 g/mol
D-Glucose			180.16 g/mol
D-Maltose	Disaccharide	Glucose + Glucose	342.30 g/mol
D-Melibiose		Glucose + Galactose	342.30 g/mol
Sucrose		Glucose + Fructose	342.30 g/mol
D-Raffinose	Trisaccharide	Glucose + Galactose + Fructose	504.42 g/mol
Amylopectine	Polysaccharide	α (1→4) and α (1→6) glycocidic bonds	variable
Amylose		Fewer α (1→6) glycocidic bonds	variable
Dextrin			variable

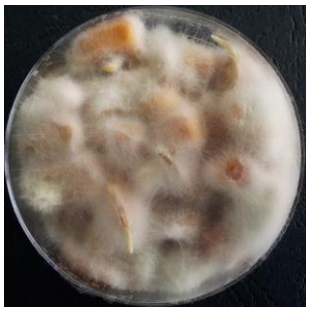
Fatty acids

	Type	Molecular mass
Linoleic acid	18:2 lipid number	280.45 g/mol
Oleic acid	18:1 lipid number	282.46 g/mol
Palmitic acid	16:0 lipid number	256.42 g/mol

APPENDIX K: Default requisites for designing primers and probes for quantitative real-time PCR assays (adapted from Rodríguez *et al.*, 2015)

REQUISITES	PRIMERS	PROBES
GC content	30–80%	
Calculated T_m	50–60°C (always > 55°C)	8–10°C above T_m primer
Runs of identical nucleotides	Maximum 3 (No G bases)	
Primer/probe length	15–30 bp	
PCR product length	50–150 bp (optimum < 80 bp)	
Distance forward primer to probe	50 bp	
Primer-dimers, hairpins	Avoid	Avoid
3′ end rule (3′ instability)	Maximum 2 G or C in the last 5 bp	–
Autoquenching	–	No G on the 5′ end
GC ratio	–	C > G
Degree of degeneracy of bases	Avoid	Avoid

APPENDIX L: Hyphal growths of *F. verticillioides* FV1 on irradiated maize kernels at 0.95 and 0.98 a_w after 5, 7, 10 and 14 days incubation at 25°C

	0.95 a_w	**0.98 a_w**
Day 5		
Day 7		
Day 10		
Day 14		

APPENDIX M: Hyphal growth of *F. verticillioides* (FV1), *F. verticillioides* + *Clonostachys rosea* 016 (FV1+BCA1) and *F. verticillioides* + Gram-negative rod (FV1+BCA5) on maize cobs (*in planta*) of different ripening stages after 10 days incubation

At 25 and 30°C, all treatments (FV1, FV1+BCA1, FV1+BCA5) showed fungal growth. FV1 and FV1+BCA5 showed similar colony appearance of *F. verticillioides* (cottony, salmon-coloured). Quite possibly the Gram-negative rod (BCA5) showed little or no inhibition at all of FV1. On the contrary, FV1+BCA1 only showed (whitish, velvety) colony appearance of *Clonostachys rosea* 016 (BCA1) and no/little growth of FV1.

At 35°C, no fungal growth was visible at all treatments (FV1, FV1+BCA1, FV1+BCA5). This might indicate that 35°C is not suitable for growth of both pathogen and biocontrol.

Furthermore, at 35°C + 1,000 ppm carbon dioxide (CO_2), no fungal growth was visible at all treatments (FV1, FV1+BCA1, FV1+BCA5). However, overgrowth of *Aspergillus flavus* was observed on all maize cobs. This was due to the wider temperature range that permitted growth of *A. flavus* which might indicate that control of *F. verticillioides* at climate change might be pointless as FV1 will easily be outgrown by *A. flavus*.